DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF FORMOTEROL FUMARATE AND BUDESONIDE IN THEIR COMBINED DOSAGE FORM

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY,

CHENNAI – 600 032

In partial fulfillment of the requirements for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted By

KUMAR .A

Reg. No:261630702

Under the guidance of

Dr. M.CHITRA, M. Pharm., Ph.D.,

Associate Professor

DEPARTMENT OF PHARACETICAL ANALYSIS

JKKMMRF'S – ANNAI JKK SAMPOORANI AMMAL

COLLEGE OF PHARMACY,

KOMARAPALAYAM – 638 183

OCTOBER– 2018

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LIST OF ABBREVIATIONS AND UNITS

%	Percentage
% RSD	Percentage Relative Standard Deviation
Hg	Microgram
Pi	Microliter
µm	Micrometer
API	Active Pharmaceutical Ingredient
AAS	Atomic Absorption Spectroscopy
Avg,	Average
AMD	Automated Multiple Development
Cm	Centimeter
CI	Confidence Interval
DAA	Direct-Acting Antiviral Agent
DSC	Differential Scanning Colorimetry
DMSO	Dimethyl Sulfoxide
DMF	Dimethyl Formamide
GC	Gas Chromatography
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HSTLC	High Speed TLC
ICH	International Conference on Harmonization
id	Internal Diameter

IR	Infra-Red
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
mg	Milligram
ml	Milliliter
mm	Millimeter
min	Minutes
ng	Nano gram
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NMT	Not more than
ODS	Octa Decyl Silane
OPLC	Over Pressured Layer Chromatography
R	Regression coefficient
r^2	Correlation coefficient
Rf	Retention factor
RP- HPLC	Reverse Phase High Performance Liquid Chromatography
RPL	Rotation Planer Chromatography
SD	Standard Deviation
SE	Standard Error
SFC	Super Critical Fluid Chromatography
S.NO	Serial number
TLC	Thin Layer Chromatography
UV	Ultraviolet Spectrophotometer

1. INTRODUCTION

Analysis may be defined as the science and art of determining the composition of materials in terms of the elements or compounds contained in them. In fact, analytical chemistry is the science of chemical identification and determination of the composition (atomic, molecular) of substances, materials and their chemical structure[1].

Chemical compounds and metallic ions are the basic building blocks of all biological structures and processes which are the basis of life. Some of these naturally occurring compounds and ions (endogenous species) are present only in very small amounts in specific regions of the body, while others such as peptides, proteins, carbohydrates, lipids and nucleic acids are found in all parts of the body. The main object of analytical chemistry is to develop scientifically substantiated methods that allow the qualitative and quantitative evaluation of materials with certain accuracy. Analytical chemistry derives its principles from various branches of science like chemistry, physics, microbiology, nuclear science and electronics. This method provides information about the relative amount of one or more of these components.

According to WHO, a drug may be defined as any substance or product that is used or intended to be used for modifying or exploring physiochemical systems or pathophysiological stages for the benefit of the patient. In today's world, drugs have become an inevitable part of one's life. We use drugs almost every single day. The quality of the drug is the most essential feature which directly affects the life of the customer. The quality of any product can be judged by only analyzing it. This investigative process is a part of science which is called as analysis. Analytical

chemistry is the science of obtaining, processing and communicating information about the composition and structure of matter.

Every country has legislation on bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called "Pharmacopoeia" (e.g. IP, USP, and BP). Quantitative chemical analysis is an important tool to assure that the raw material used and the intermediate products meet the required specifications. Every year number of drugs is introduced into the market. Also quality is important in every product or service, but it is vital in medicines as it involves life.

There is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, report of new toxicities and development of patient resistance and introduction of better drugs by the competitors. Under these conditions standard and analytical procedures for these drugs may not be available in Pharmacopoeias. In instrumental analysis, a physical property of the substance is measured to determine its chemical composition. Pharmaceutical analysis comprises those procedures necessary to determine the identity, strength, quality and purity of substances of therapeutic importance[2].

Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw material on which degree of purity and quality of medicament depends. The quality of the drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations.

Quality control is a concept which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production. The decision to release or reject a product is based on one or more type of control action. With the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex instrumentation. Providing simple analytical procedure for complex formulation is a matter of most importance. So, it becomes necessary to develop new analytical methods for such drugs. In brief the reasons for the development of newer methods of drugs analysis are

The drug or drug combination may not be official in any pharmacopoeias.

- A proper analytical procedure for the drug may not be available in the literature due to Patent regulations.
- Analytical methods for a drug in combination with other drugs may not be available.

1.1 DIFFERENT METHODS OF ANALYSIS[3]

The following techniques are available for separation and analysis of components of interest.

- **Spectral methods**

The spectral techniques are used to measure electromagnetic radiation which is either absorbed or emitted by the sample.

E.g. UV-Visible spectroscopy, IR spectroscopy, NMR, ESR spectroscopy, Flame photometryFluorimetry.

❖ **Electro analytical methods**

Electro analytical methods involved in the measurement of current voltage or resistanceas a property of concentration of the component in solution mixture. E.g. Potentiometry, Conductometry, Amperometry.

❖ **Chromatographic methods**

Chromatography is a technique in which chemicals in solutions travel down columns or over surface by means of liquids or gases and are separated from each other due to their molecular characteristics.

E.g. Paper chromatography, thin layer chromatography (TLC), High performance thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC), Gas chromatography (GC).

❖ **Miscellaneous Techniques**

Mass Spectrometry, Thermal Analysis.

- **Hyphenated Techniques**

GC-MS (Gas Chromatography – Mass Spectrometry), LC-MS (Liquid Chromatography – Mass Spectrometry), ICP-MS (Inductivity Coupled Plasma- Mass Spectrometry), GC-IR (Gas Chromatography – Infrared Spectroscopy), MS-MS (Mass Spectrometry – Mass Spectrometry).Analytical techniques that are generally used for drug analysis also include Analytical methods for the quantitation of the drug in biological fluids may not be available.

The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and

these may not be reliable. biological and microbiological methods, radioactive methods and physical methods etc. are mentioned in Table 1.1.

Table 1.1**:** Summary of Hyphenated separation techniques[4]

Separation technique	**Hyphenated mode**
Liquid chromate graphy	Liquid chromatography-mass spectrometry(LC/MS) Liquid chromatography-Fourier-transform infrared Spectrometry(LC-FTIR) Liquid chromatography-nuclear magnetic resonance spectroscopy(LC/NMR) Liquid chromatography-inductively coupled plasma mass spectrometry(LC-ICPMS)
Gas chromat ography	Gas chromatography-mass spectrometry(GC/MS) Gas chromatography-Fourier-transform infrared(GC-FTIR) Gas chromatography-FTIR-MS(GC-FTIR-MS)
Capillary electrophoresis	Capillary electrophoresis-mass spectrometry(CE/MS) Capillary electrophoresis- nuclear magnetic resonance spectroscopy(CE/NMR) Capillary electrophoresis-surface enhanced Raman spectrometry (TLC-SERS)
Thin layer chromatography(T LC)	Thin layer chromatography- mass spectrometry(TLC/MS) Thin layer chromatography- surface enhanced Raman spectrometry(TLC-SERS)
Superfic ial fluid chromat ography/ extractio n(SFC/S FE)	Superficial fluid extraction-capillary gas chromatography-mass spectrometry(SFE-CGC-MS) Superficial fluid-Fourier-transform infrared(SFC-FTIR)

1.2 INTRODUCTION TO HPLC[1]

HPLC is also called as high pressure liquid chromatography since high pressure is used to increase the flow rate and efficient separation by forcing the mobile phase through at much higher rate .The pressure is applied using a pumping system. The development of HPLC from classical column chromatography can be attributed to the development of smaller particle size .Smaller particle size is important since they offer more surface area over the conventional large particle sizes. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages.

- Improved resolution of separated substances column packing with very small (3,5 and 10 µm) particles Faster separation times (minutes)
- Sensitivity
- Reproducibility
- Continuous flow detectors capable of handling small flow rates Easy sample recovery, handling and maintenance.

1.2. Types of HPLC Techniques[5]

1.2.1 Based on Modes of Chromatography

These distinctions are based on relative polarities of stationary and mobile phases

- **Reverse phase chromatography*:***In this the stationary phase is non-polar and mobile phase is polar. In this technique the polar compounds are eluted first andnon polar compounds are retained in the column and eluted slowly. Therefore it is widely used technique.

- **Normal phase chromatography:** In this the stationary phase is polar and mobile phase is non-polar. In this technique least polar compounds travel faster and are eluted first where as the polar compounds are retained in the column for longer time and eluted.[4]

1.2.2 Based on Principle of Separation

- **Liquid/solid chromatography (Adsorption*):***LSC, also called adsorption chromatography,the principle involved in this technique is adsorption of the components onto stationary phase when the sample solution is dissolved in mobile phase and passed through a column of stationary phase.The basis for separation is the selective adsorption of polar compounds; analytes that are more polar will be attracted more strongly to the active silica gel sites. The solvent strength of the mobile phase determines the rate at which adsorbed analytes are desorbed and elute. It is widely used for separation of isomers and classes of compounds differing in polarity and number of functional groups. It works best with compounds that have relatively low or intermediate polarity.

- **Liquid/Liquid chromatography (Partition Chromatography*):***LLC, also called partition chromatography, involves a solid support, usually silica gel or kieselguhr, mechanically coated with a film of an organic liquid. A typical system for NP LLC column is coated with ß, ß'-oxy dipropionitrile and a

nonpolar solvent like hexane as the mobile phase. Analytes are separated bypartitioning between the two phases as in solvent extraction. Components more soluble in the stationary liquid move more slowly and elute later.

- **Ion exchange:** In this the components are separated by exchange of ions between an ion exchange resin stationary phase and a mobile electrolyte phase. A cation exchange resin is used for the separation of cations and anion exchange resin is used to separate a mixture of anions.

- **Size exclusion:**In this type, the components of sample are separated according to their molecular sizes by using different gels (polyvinyl acetate gel, agarose gel).ex: separation of proteins, polysaccharides, enzymes and synthetic polymers.

- **Chiral chromatography:** In this type of chromatography optical isomers are separated by using chiral stationary phase.

- **Affinity chromatography:** In this type, the components are separated by an equilibrium between a macromolecular and a small molecule for which it has a high biological specificity and hence affinity.

1.2.3 Based on elution technique[6]

- **Isocratic separation**: In this technique, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained throughout the process.

- **Gradient separation:** In this technique, a mobile phase combination of lower polarity or elution strength is followed by gradually increasing polarity or elution strength.

1.2.4 Based on the scale of operation

- **Analytical *HPLC*:**Where only analysis of samples are done. Recovery of samples for reusing is normally not done, since the sample used is very low. Ex: µg quantities.

- **Preparative *HPLC:*** Where the individual fractions of pure compounds can be collected using fraction collector. The collected samples are reused. Ex: separation of few grams of mixtures by HPLC.

1.2.5.Based on type of analysis

- **Qualitative analysis**: Which is used to identify the compound detect the presence of impurities to find out the number of components. This is done by using retention time values.

- **Quantitative analysis*:***This is done to determine the quantity of individual or several components of mixture. This is done by comparing the peak area of the standard and sample.

1.3. INSTRUMENTATION OF HPLC[7]

The basic liquid chromatograph consists of six basic units. The mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results.

Mobile phase (solvent) reservoirs and solvent degassing

The mobile phase supply system consists of number of reservoirs (200mL to 1,000mL in capacity). They are usually constructed of glass or stainless steel materials which are chemically resistant to mobile phase**.**

Mobile phase

Mobile phases in HPLC are usually mixtures of two or more individual solvents. The usual approach is to choose what appears to be the most appropriate column, and then to design mobile phase that will optimize the retention and selectivity of the system. The two most critical parameters for nonionic mobile phases are strength and selectivity.

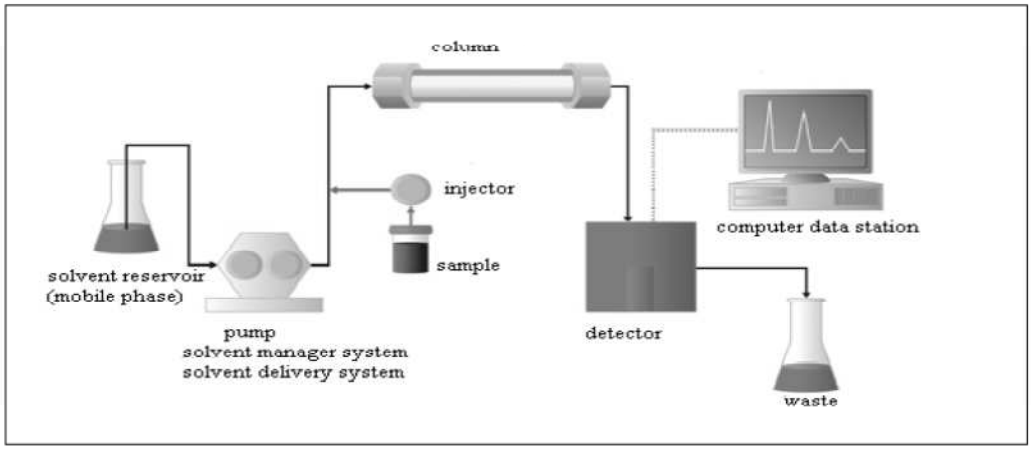

Figure 1.1.Compnents of HPLC Instrument block diagram.

- **Mobile phase preparation**

Mobile phases must be prepared from high purity solvents, including water that must be highly purified. Mobile phases must be filtered through ≤1 µm pore size filters and be degassed before use.

- **Degassing of solvents**

Many solvents and solvent mixtures (particularly aqueous mixtures) contain significant amounts of dissolved nitrogen and oxygen from the air. These gasses can form bubbles in the chromatographic system that cause both serious detector noise and loss of column efficiency. These dissolved gases in solvent can be removed by the process of degassing. Every solvent must be degassed before introduction into pump as it alter the resolution of column and interfere with monitoring of the column effluent.

Degassing is done in many ways:

- By warming the solvents
- By stirring vigorously with a magnetic stirrer
- By subjecting to vaccum filtration
- By ultra sonication (using ultrasonicator)
- By bubbling He gas through the solvent reservoir.

Table 1.2**:**Physical properties of common HPLC solvents[8].

Solvent	**MW**	**BP**	**RI (25^{o}C)**	**UVCut-off(nm)**	**Density g/mL(25^{o}C)**	**Viscosity (25^{o}C)**	**Dielectric Constant**
Acetonitrile	41.0	82	1.342	190	0.787	0.358	38.8
Dioxane	88.1	101	1.420	215	1.034	1.26	2.21
Ethanol	46.1	78	1.359	205	0.789	1.19	24.5
Ethylacetate	88.1	77	1.372	256	0.901	0.450	6.02
Methanol	32.0	65	1.326	205	0.792	0.584	32.7
CH_2Cl_2	84.9	40	1.424	233	1.326	0.44	8.93
Isopropanol	60.1	82	1.375	205	0.785	2.39	19.9
n-propanol	60.1	97	1.383	205	0.804	2.20	20.3
THF	72.1	66	1.404	210	0.889	0.51	7.58
Water	18.0	100	1.333	170	0.998	1.00	78.5

1.3.2 Pumping systems

The pumping system is one of the most important features of an HPLC system. There is a high resistance to solvent flow due to the narrow columns packed with small particles and high pressures are therefore required to achieve satisfactory flow rate. The main requirements of pumping systems are:

- Generation of pressures up to 6000 psi.
- Pulse free output
- Flow rates ranging from 0.01 to 10mL/min
- Flow control and flow reproducibility of ± 0.5%
- Corrosion resistant components (seals of Teflon and stainless steel)
- Should be easy to dismantle and repair.

There are three basic types of pumps in common use[9].

1. Reciprocating pumps.
2. Displacement pumps or syringe pumps.
3. Pneumatic pumps or constant pressure pumps.

1.3.3 Sample introduction system

Injection ports are of two basic types,

The sample is injected directly into the column.

The sample is deposited before the column inlet and then swept by a valving action into the column by the mobile phase.

Injectors should provide the possibility of injecting the liquid sample within the range of 0.1 to 100 mL of volume with high reproducibility and under high pressure (up to the 4000psi).They should also produce minimum band broadening and minimize possible flow disturbances. The most useful and widely used sampling device for

modern LC is the micro sampling injector valve. With these sampling valves, samples can be introduced reproducibly into pressurized columns without significant interruption of flow, even at elevated temperatures. High-performance valves provide extra column band-broadening characteristics comparable or superior to that of syringe injection.

1.3.4 Columns

Typical analytical columns are 10, 15 and 25 cm in length and are fitted with extremely small diameter (3, 5 or 10μm) particles. The internal diameter of the columns is usually 4 or 4.6 mm; this is considered the best compromise among sample capacity, mobile phase consumption, speed and resolution. Preparative columns are of larger diameter. Packing of the column tubing with the small diameter particles requires high skill and specialized equipment. For this reason, it is generally recommended that the most experienced chromatographers purchase prepacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment. The column can be classified based on the material bonded to the silica packed surface such as C_4, C_8, C_{18}, phenyl, chiral, cyanomicrobore columns (1mm to 100cm), U shaped and coiled columns are available. Guard columns are used before the analytical column to increase the life of analytical columns by retaining non eluted components and particulate matter.

Table 1.3: Column Selection Flow Chart[10].

Sample	**LC mode**	**Column choice**
Basic or acidic	Reverse Phase-ion pair (allows neutral and charged Compounds To be simultaneously analyzed)	C_{18}, C_8, C_6, C_4, C_2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13).
Ionisable	Ion Exchange Anionic, cationic	Strong cations exchange, Strong anion exchange.
Neutral	a)Normal phase b)Reverse phase	a)Increasing the polarity of bonded phases diol, CN, NH2, silica alumina b) C18, C_8, phenyl, C2.

- **Column Thermostats**

Control of column temperature is important in liquid chromatography. The effect of temperature on retention times and reproducibility is quite significant, especially when using the reverse phase models.

1.3.5 Detectors[11]

Optical detectors are most frequently used. These detectors pass a beam of light through the flowing column effluent as it passes through a low volume (~ 10mL) flow cell. The most commonly used detector in LC is the ultraviolet absorption detector. A variable wavelength detector of this type, capable of monitoring from 190 to 460-600 nm, will be found suitable for the detection of the majority samples.

Other types of Detectors:

- UV detector
- Refractive index detector
- Flourimetric detector
- Conductivity detector
- Amperometric detector
- Photodiode array detector (PDA detector).

1.3.6 Data handling

Data handling in chromatography now ranges from a simple pen recorder to complicated computer integration and computerized data handling systems. Several manufacturers today offered microprocessor controlled chromatographs. Thus the solvent delivery system, injector, oven, detector, fraction collector and data reduction can be carried under the control of a central microprocessor with the capability to program sequential parameters.

1.4. CHROMATOGRAPHIC PARAMETERS[13]

1.4.1Capacity factor (k^1)

The capacity factor is a measure of the degree of retention of an analyte relative to an un-retained peak. It is affected by solvent composition, separation, aging and temperature of separation.

$$k1 = \frac{(t_R - t_0)}{t_0}$$

Where, t_R= retention time for the sample peak.

t_0=retention time for the unretained peak.

The peak should be well-resolved from other peaks and the void volume. Generally the value of k' is > 2.

1.4.2Resolution (Rs)

Resolution is ability of a column to separate Chromatographic peaks. Resolution can be improved by increasing column length, decreasing particle size, increasing temperature, changing the eluent or stationary phase.

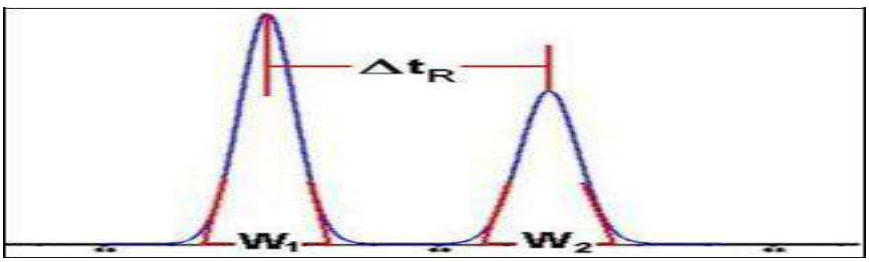

Fig. 1.2 :Resolution of peaks

$$R_S = \frac{(t_{R_1} - t_{R_2})}{0.5(t_{W_1} + t_{W_1})}$$

Where,

t_{R1},t_{R2} are retention times of the first and second adjacent bands.

$tw_1$and $tw_2$are baseline at bandwidth of the peaks for reliable quantitationwell-separated peaks are essential for quantitation.

$R_s$of> 2 between the peak of interest and the closest potential interfering peak (impurity, excipient, degradation product, internal standard, etc.) are desirable.

1.4.3.Theoretical plate number / Efficiency (N)[12]

A measure of peak band spreading determined by various methods, some of which are sensitive to peak asymmetry. Smaller the band spread higher the theoretical plate indicates good column and system performance. A theoretical plate is an imaginary or hypothetical unit of a column where equilibrium has been established between stationary phase and mobile phase. Theoretical plate number is a measure of column efficiency that is, how many peaks can be located per unit run-time of the Chromatogram.

$$N = 16[t/w_b]^2$$

Where t =retention time for the sample peak,

w_b= peak width.

$$H = \frac{L}{N}$$

N is fairly constant for each peak on a Chromatogram with a fixed set of operatingconditions. H (height) or HETP (height equivalent of a theoretical plate) measures the column efficiency per unit length (L) of the column. Parameters which can affect N or H include peak position, particle size in column, flow rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the analyte.The theoretical plate number depends on elution time but in general should be > 2000.

1.4.4 Tailing factor (T) or Asymmetry factor (AF)[14]

It is a measure of the symmetry of a peak.

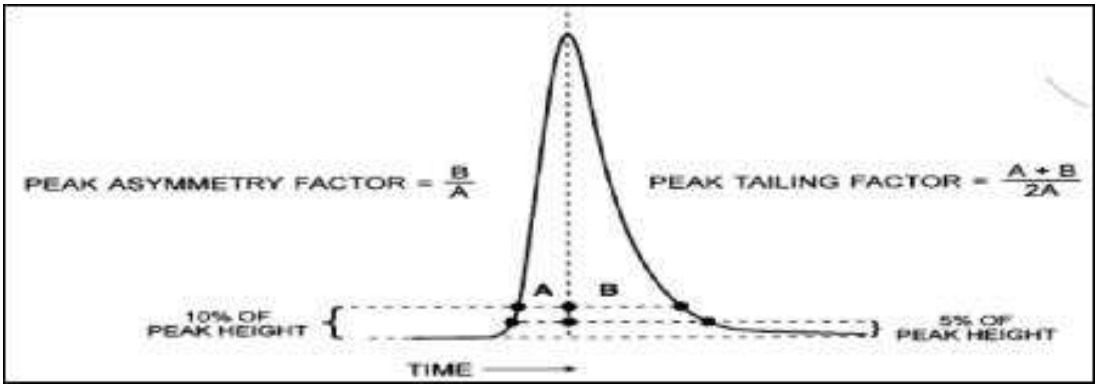

Fig. 1.3: Peak Asymmetry

$$T = \frac{W}{2f}$$

Where, W= peak width at 5 % height,

f = distance from peak front to apex point at 5% height.

The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak.

1.5. METHOD DEVELOPMENT AND VALIDATION[13]

1.5.1 Introduction to HPLC Method Development

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result .it should be simple as possible,yet it should allow the use of sophisticated tools such as computer modeling.

- ***Nature of sample***

Before beginning of method development, we need to review about the sample, in order to define goals of separation. The kinds of sample related information that can be important are:

The various kinds of sample related information that may be important for method development was summarized in Table 1.4.

Table .1.4**:**Information Concerning Sample[15]

S.NO	Sample requirements
1.	Number of compounds present
2.	Chemical structures
3.	Molecular weights of compounds
4.	pK_a values of compounds
5.	UV spectra of compounds
6.	Concentration range of compounds in samples of interest
7.	Sample solubility

- ***Method requirements***

The goals of the analytical method that need to be developed are considered. The detection limits, selectivity, linearity, range, accuracy and precision are defined.

- ***Separation goals***

The goals of HPLC separation need to be specified clearly, which include:

- The use of HPLC to isolate purified sample components for spectral identification or quantitative analysis
- It may be necessary to separate all degradants or impurities from a product for reliable content assay or not
- In quantitative analysis, the required levels of accuracy and precision should be known (a precision of 1 to 2 % is usually achievable)
- Whether a single HPLC procedure is sufficient for raw material or one or more different procedures are desired for formulations

- When the number of samples for analysis at one time is greater than 10, a run time of less than 20 minutes often will be important.

- Knowledge on the desired HPLC equipment, HPLC experience and academic training do the operators have?

- ***Literature search and prior methodology***

The information related to the analyte is surveyed for synthesis, physical and chemical properties, solubility and relevant analytical methods. Books, periodicals and USP/NF, and publications are reviewed.

- ***Choosing a method***

a) Using the information in the literatures, methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for analytes.

b) If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

- ***Instrumental setup and initial studies***

a) The required instrumentation is setup installation, operational and performance qualifications of instrumentation verified by using laboratory standard operating procedures.

b) Always new solvents, filters are used, for example, method development is never started, on a HPLC column that has been used earlier.

c) The analyte standard in a suitable injection/introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (ex: bulk drug) then it is possible to start work with the actual sample.

d) Analysis is done using analytical conditions described in the existing literature.

- ***Optimization***

During optimization one parameter is changed at a time and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan and every step is documented (in lab notebook) in case of dead ends.Reversed-Phase HPLC offers multiple parameters for optimizing a separation.

To plan separation by RP-HPLC, the analyst must select both a stationary phase and a mobile phase appropriate to the analyte under investigation. In addition the analyst must identify chromatographic conditions that will maintain the sharpness of analyte bands as the sample moves through the stationary phase column to the detector. The first attempt at optimization of separation requires selection of a promising set of conditions.

To optimize the solvent strength, one approach is to begin with a mobile phase that is probably too strong and reduce solvent strength to increase k' (capacity factor, is the measure of retention) between successive runs. When all the peaks fit within the range 0.5< k' < 20, the mobile phase is near optimum from the standpoint of solvent strength. The solvent properties are useful when it comes to selecting one or more solvents.

Table 1.5. Shows preferred experimental conditions for the initial HPLC separation[16].

Separation Variable	**Preferred Initial Choice**
Column	
Dimensions(length,ID)	15 × 0.46 cm
Particle size	5 µm [a]
Stationary phase	C8 or C18
Mobile phase	
Solvents A and B	Buffer – acetonitrile
%B	80-100 % [b]
Buffer (compound, P^H, concentration)	25Mmpotassium phosphate, 2.0 < pH < 3.0 [c]
Additives (eg., amine modifiers, ion pair reagents)	Do not use initially
Flow rate	1.5 -2.0 mL/min
Temperature	35-45 ^{0}C
Sample size	
Volume	<25 µL
Weight	<100 µg

- 3.5 m particles are an alternative, using a 7.5 cm column.
- For an initial isocratic run; an initial gradient run is preferred.
- No buffer required for neutral samples; for pH <2.5, pH-stable columns recommended.

Once the solvent strength has been properly adjusted for the sample, the next selection parameter that can be explored is α (band spacing). In many cases, it is possible to obtain a satisfactory separation simply by varying solvent strength. This is usually sufficient for simple and easily resolved samples such as less polar and medium polar non-ionic solutes, but for samples containing ionic and ionisable compounds, apart from changes in solvent strength, several other separation variables are to be altered to optimize band spacing, retention or peak shape. Some of the more commonly used variables are listed in Table 1.6.

Table1.6**:** Variables for improving separation[17]

Variable	**Comment**
Choice of organic solvent	A change from methanol to acetonitrile or THF often results in large changes in separation.
Mobile phase pH	A Change in pH may result in a major effect on band spacing for samples that contain ionic or ionisable compounds.
Solvent strength	A change in percent organic often provides significant changes in retention and separation.
Column type	This refers to the choice of bonded-phases for reversed-phase LC (C_{18}, C_8,Phenyl,cyano etc)
Concentration of mobile phase additives	The most common additives for varying band spacing include amine modifiers, acid modifiers, buffers and salts.
Temperature	The temperature can be varied between 0 to 70ºC for the purpose of controlling band spacing; however, temperatures of 25-60 ºC are more common.

For selectivity changes, it is sometimes desirable to substitute one organic solvent for another. The solvent strength monograph provides required information for such substitution of solvent in reversed-phase mobile phases.Stationary phase optimization is also an option for reversed-phase LC. Octadecyl and octyl are the most commonly used phases, but cyano and phenyl columns are also frequently used. Mobile phase optimization experiments should be performed for each column to be tested to obtain the desired separation.

For the samples that contain acidic or basic compounds, retention may vary with both the ionic strength and pH of the buffer. If the pKa values are known for a group of compounds to be separated by RP-HPLC, and if these pKa values are different, then it is likely that a pH near (±2 units) the average pKa value of the mixture should provide good separation.The recommended additives for the reversed-phase mobile phases in the case of samples that contain acids, bases or salts are given in Table 1.7.

Table1.7**:** Recommended additives for reversed-phase mobile phases[18].

Sample characteristics	**Additives**
Basic compounds (ex: amines)	50mM Phosphate buffer, 30 Mmtriethylamine (buffer pH 3.0)
Acidic compounds (ex: carboxylic acids)	50mM Phosphate buffer, 1% acetic acid (buffer pH 3.0)
Mixture of acids and bases	50mM Phosphate buffer, 30 mMtriethylamine, 1% acetic acid
Cationic salts (ex: tetralkylquarternary ammonium compounds)	30 mMtriethylamine, 50mM sodium nitrate
Anionic salts	1% acetic acid, 50mM sodium nitrate

A temperature of 40-60^{0}C appears to be convenient in reversed phase-LC, when sample component permits. Compared to ambient conditions operation at these temperatures usually doubles the column efficiency in terms of number of theoretical plates.Different concentrations of the various organic solvents are required to maintain constant solvent strength. Varying the pH of mobile phase may drastically alter separation selectively in RP-HPLC, if the sample components are acids or bases. When dealing with a sample component of weak base, pKa 8 the ion suppression technique can be used by adding an acidic buffer. If this is ineffective, an ion pair reagent, such as alkyl sulfonic acid may be added. For strong bases, pKa>8, ion pairs are more effective than ion suppression.Mobile phase composition has a major effect on band spacing; no other variable will generally prove as powerful for controlling values of separation. The separation goals in HPLC method development are summarized in Table 1.8.

Any molecule has either n, π or σ or a combination of these electrons. These bonding (σ& π) and nonbonding (n) electrons absorb the characteristic radiation and undergoes transition from ground state to excited state. In general, non-bonding lone pair of electrons of hetero atoms is the least strongly bound in a molecule and in the bonding levels π – electrons have higher energy levels than the corresponding σ – electrons whilst in the anti-bonding levels the order is reversed.

Table 1.8**:**Separation goals in HPLC method development[19].

Goal	**Comment**
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	<5-10 min is desirable for routine procedures.
Quantitation	2% for assays; 5% for less-demanding analyses 15% for trace analyses.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratios.
Solvent Consumption	Minimum mobile-phase use per run is desirable.

Documentation of analytical figures of merit

The originally determined analytical figures of merit Limit of Quantitation (LOQ), Limit of Detection (LOD), Linearity, time per analysis, cost, sample preparation etc., are documented.

Evaluation of method development with actual samples

The sample solution should lead to absolute identification of the analyte peak of interest apart from all other matrix components.

Determination of percent recovery of actual sample and demonstration of quantitative sample analysis

- Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average ± standard deviation) from sample to sample and whether recovery has been optimized is determined. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainity.

- The validity of analytical method can be verified only by laboratory studies. Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended applications.

Table .1.9: Steps involved in development of HPLC method[20]

Literature survey: To avoid duplication of the method and invention of new method.Information about the solubility, the structure of the drugs and their Physico-chemical properties for the known compounds and can be applied for the new compounds.

↓

Selection of chromatographic method: First reversed phase should be tried.If not successful, normal phase should be taken into consideration.For ion exchange or ion pair chromatography, first ion suppression by pH control and reversed phase chromatography should be tried.

↓

. **Selection of stationary phase:** Matching the polarity of sample and stationary phase and using a mobile phase of different polarity achieves a successful separation

↓

Selection of mobile phase: Reversed phase bonded packing, when used in conjunction with highly polar solvents. Mobile phase may be either single liquid or combination of liquids, which are compatible with sample, column and instrument.

↓

Selection of suitable detector: Detector is the eye of HPLC system and measures the compounds after their separation on the column. There are basically two types of detectors: bulk property detectors; Solute property detectors.UV detector is the first choice because of its convenience and applicability in case of most of the samples. The latest version of equipment is available with photo diode-array detectors.

1.6. ANALYTICAL METHOD VALIDATION[21]

Method validation as per ICH can be defined as "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".

- **Objective of validation**

There are two important reasons for validating assays in the pharmaceutical industry.The first, and by for most important is that assay validation is an integral part of the quality control system.The second is that current good manufacturing practice regulation requires assay validation. In industry it would be difficult to confirm that the product being manufactured is uniform and that meet the standards set to assure fitness for use. The varying nature of the differences between the analytical development laboratory and quality control laboratory is a good reason for validation program.

Method validation study includes Specificity / Selectivity, Linearity, Accuracy, Precision, Limit of detection, Limit of Quantitation, Robustness, System suitability and Stability criteria.

- **Accuracy**

The accuracy of measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value should ideally be identical to the true

value.Typically, accuracy is represented and determined by recovery studies but there are three ways to determine accuracy:

Comparison to a reference standard

Recovery of the analyte spiked into blank

matrix or Standard addition of the analyte.

The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentrations levels the specified range (i.e., three concentrations and three replicates of each concentration). Accuracy was tested (%Recovery and %RSD of individual measurements) by analyzing samples at least in triplicate, at each level (80%, 100% and 120% of label claim) is recommended. For each determination fresh samples were prepared and assay value is calculated. Acceptance criteria:T he accuracy should be within 98-102%.

- **Precision**

Precision can be defined as "the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample". A more comprehensive definition proposed by the International Conference on Harmonization (ICH) divides precision into three types:

- Repeatability
- Intermediate
- precision
- Reproducibility

- **Repeatability:** Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision and involves multiple measurements of the same sample (different preparations) by the same analyst under the same conditions. At least 5 or 6 determinations

At two or three different concentrations should be done and the relative standard deviations were calculated. The % RSD can be calculated by,

$$\% \, \mathbf{RSD} = \frac{\mathbf{SD} \times \mathbf{100} \, \%}{\mathbf{MEAN}}$$

Where, RSD = relative standard deviation

SD = standard deviation

The standard deviation SD is given by,

$$\sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - x)^2}$$

- **Intermediate precision*:*** It is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory. Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment etc.

- **Reproducibility:**Reproducibility expresses the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Acceptance criteria*:*The% RSD should be less than 2.

- **Linearity**

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample.

It is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data are then processed using a linear least squares regression. The Resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

Acceptance criteria:Correlation coefficient(R^2)>0.998

- **Specificity**

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present; these include impurities, degradants and matrix etc. Lack of specificity of an individual analytical procedure

may be compensated by other supporting analytical procedures. This definition has the following implications:

Identification: To ensure the identity of an analyte.

Purity Tests: To ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content etc.

Assay (content or potency): To provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

Acceptance criteria: No interference should be present.

- **Limit of Detection**

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD can be defined as the smallest level of analyte that gives a measurable response. The detection limit is usually expressed as the concentration of the analyte (percentage parts per million) in the sample. It is usually determined by 3 ways:

- Based on Visual Evaluation
- Based on Signal-to-Noise
- Based on the Standard Deviation of the Response and the slope
- The limit of detection may be expressed as

$$\mathbf{LOD} = \frac{\mathbf{3.3\sigma}}{\mathbf{S}}$$

Where, σ = the standard deviation of the

response S = the slope of the calibration

curve.

Acceptance criteria*:*S/N should be 3:1

- **Limit of Quantitation**

The quantitation limit of an analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ is usually expressed as the concentration of the analyte (percentage parts per million) in the sample. . It is usually determined by 3 ways:

- Based on Visual
- Evaluation Based on Signal-to-Noise
- Based on the Standard Deviation of the Response and the slope

The limit of Quantitation may be expressed as:

$$\mathbf{LOQ} = \frac{\mathbf{10\sigma}}{\mathbf{S}}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve.

Acceptance criteria:S/N should be 10:1

- **Robustness**

It is defined as a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.It involves a number of method parameters that are varied within a realistic range and the quantitative influence of the variables is determined. The typical variations are:

- Influence of variations of pH in a mobile phase.
- Influence of variations in mobile phase composition.
- Different columns (different lots and/or suppliers).
- Temperature.
- Flow rate.

- **System Suitability Testing**

Prior to the analysis of samples each day, the operator must establish that the HPLC system and procedure are capable of providing data of acceptable quality. This is accomplished with system suitability experiments, which can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision.

The parameters that can be used to determine system suitability prior to analysis, includes Plate number (N), Tailing factor, k and/or α, Resolution (Rs)and Relative standard deviation (RSD) of peak height or peak area for repetitive injections. Typically at least two of these criteria are required to demonstrate system suitability for any method.

Table 1.10**:** Acceptance criteria of validation for HPLC[22]

S.No	Characteristics	Acceptance criteria
1	Accuracy	98-102%
2	Precision	RSD<2
3	Specificity	No interference
4	Detection limit	S/N >3:1
5	Quantitation limit	S/N > 10:1
6	Linearity	R^2> 0.998
7	Range	80-120%

- **Stability Indicating Method**

Stability-indicating methods according to 1987 guideline were defined as the 'quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.'

This definition in the draft guideline of 1998 reads as: 'Validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference.'

The stability-indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. With the advent of International

Conference on Harmonisation (ICH) guidelines, the requirement of establishment of stability-indicating assay method (SIAM) has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light,oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products.

- **Forced degradation studies**

Forced Degradation is theStudy designed to intentionally degrade a drug substance or drugproduct under different stress conditions like acid, base, oxidation, Heat, light, moisture.

Forced degradation studies are indispensable in the development of stability-indicating and degradant-monitoring methods as part of a validation protocol. Forced degradation studies also provide invaluable insight in investigating degradation products and pathways of drug substances and products. Even though the ICH and FDA guidance documents only call for the inclusion of these studies in Phase III of the regulatory submission process, it is strongly recommended these studies be started as early as possible to be able to provide valuable information that can be used to assess the inherent stability of a drug, and to improve formulations and the manufacturing process.

Given that no specific set of conditions will be applicable to all drug substances and products, the pharmaceutical scientist should ensure the stress conditions are consistent with product decomposition under normal manufacturing, storage, and intended use conditions. Recommended stress factors include high and

low pH, elevated temperature, photolysis, and oxidation. Care should be taken to avoid under stressing or unduly over-stressing the drug substance or product, for this may lead to aberrant and non-representative results. A degradation level of approximately 10% of the drug substance should be optimal for method optimization.

- **Forced Degradation Studies are performed**

  - To understand the reactive chemistry of the drug substance
  - Help anticipate future stability issues of both drug substance and drug product Provides useful information for formulation and stability
  - Generate a sample for development of stability indicating methods for formal stability study support

Table 1.11*:* Example Conditions for Forced Degradation[23]

Study	**Condition**
Acidic	0.1N HCl
Basic	0.1N NaOH
Oxidation	3% H_2O_2
Neutral	pH - 7 Phosphate Buffer
Photolysis (UV)	1000 Watt h/M2
Photolysis (Fluorescence)	6x106 lux h

Goal: Degrade API up to 5-10%.

Stability Indicating Method (SIM): A validated method that can accurately and precisely quantitative the decrease of the API content due to degradation.

- Is specific for the drug substance
- Shows a decrease in assay value (correlated to drug substance loss) due to degradation
- Has no interference from excipients, impurities or degradation products
- Detects and quantities impurities and degradation products

FDA Comments on Peak Purity[24]

The purity testing provided was based on one wavelength and on one lot. This is not adequate to assess peak purity. Typically, the entire UV-Vis spectra (with consideration for solvent and noise controls) is analyzed at various points in the peak area (especially at leading and trailing edge) and at varying concentrations to indicate purity. There is chromatographic software to perform this type of analysis.

In addition, other instruments and techniques are used to assess peak purity. We are unable to fully evaluate the purity of the compound based solely on the information provided.

Role of Photodiode Array Detector in LC Method Development/Validation[25]

- Spectral Contrast Software For Peak Tracking and Identification
- Potential For Peak Purity System Suitability Criteria
- Peak Purity or Homogeneity Information Indicative of Co-Elution

What is needed for PDA Peak Purity Calculations?

- Compounds must have UV Absorbance
- Some Degree of Chromatographic Resolution
- Some Degree of Spectral Differences Between Compounds.

Stability indicting method is needed

- To support long term stability testing
- Demonstrate how the quality of the drug substance or product changes over time in response to environmental factors like
- Temperature
- Humidity
- Light
- Establishes storage and packaging conditions.

General points to consider

Some basic points to note in the test method are:

- The sample and standard should be dissolved in the mobile phase. If that is not possible, then avoid using too high a level of the organic solvent as compared to the level in the mobile phase.
- The sample and standard concentrations should be close if not the same.

- The samples should be bracketed by standards during the analytical procedure.

- Filtration of the samples before injection is occasionally observed Filtration Will remove particulates (centrifugation performs the same function) that may clog columns.

- Adhesion of the analyte to the filter can also happen. This will be of importance especially for low-level impurities. Data to validate this aspect should be submitted by the applicant.

2. LITERATURE REVIEW

Bhargav.Y.et al[26],(2013)A new, simple, accurate, rapid, precise RP-HPLC method was developed for the simultaneous estimation of ofloxacin and tinidazole in bulk and pharmaceutical dosage form. A good chromatographic separation was achieved with Intersil ODS C18 250×4.6mm.5µ) column and ammonium acetate buffer pH 4.0, acetonitrile, and tetrahydrofuran 60:30:10 was used as mobile phase at a flow rate of 1.0ml/min and the detection was carried out at a wavelength of 304 nm. The retention times was found to be 2.350 min for ofloxacin and 3.613 min for tinidazole. The linearity of the method was in the concentration range of 15-35µg/mL for ofloxacin and 45-105µg/mL for tinidazole. The developed method was validated for system suitability, specificity, precision, recovery and linearity according to ICH guidelines. The method was successfully applied for routine analysis for the determination of oflaxacin and tinidazole in bulk and dosage forms.

Pankaj.B et al[27],(2012) A simplereverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of Ofloxacin and Ornidazole in infusion dosage form. The separation was carried out using a mobile phase containing methanol and buffer (equal proportion of 0.01M orthophosphoric acid and 0.01M sodium phosphate monobasic dihydrate) with pH 4.00 adjusted by 20% of triethylamine in the ratio of 60:40 v/v. The column used was HiQSil C18 (150 mm x 4.6mm i.d, 5 µ) with flow rate of 1 mL / min using UV detection at 300 2 nm. The described method was linear over a concentration range of 1.25-10 µg/mL (r >0.9991) forOfloxacin and 3.12-25 µg/mL (r >0.9992) for Ornidazole. Separation was achieved within 5 min.

The mean % recovery was found to be 99.94% for Ofloxacin and 100.27 % for Ornidazole. The limit of detection (LOD) for Ofloxacin and Ornidazole were found to be 0.146 and 0.25 µg/mL respectively. Whereas, the limit of quantification (LOQ) for Ofloxacin and Ornidazole was 0.44 and 0.77 µg/mL respectively.

Vasudevan.Metal et al[28], (2009) A simple Reverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of Ofloxacin and Tinidazole in combination. The separation was carried out using a mobile phase consisting of 0.5%v/v Triethylamine buffer of pH 3.0 and Acetonitrile in the ratio of 73: 27. The column used was KromasilC , 5µ, 15 cm × 4.6 mm id with flow rate of 1.2 ml / min using PDA detection at 303 nm. The described method was linear over a concentration range of 10-50 µg/ml and 30-150 µg/ml for the assay of Ofloxacin and Tinidazole respectively. Ambroxol (50 µg/ml) was used as internal standard. The retention times of Ofloxacin, Tinidazole and Ambroxol were found to be 2.3, 4.1 and 5.1min respectively. Results of analysis were validated statistically and by recovery studies. The limit of quantification (LOQ) for Ofloxacin and Tinidazole were found to be 10 and 30 µg/ml respectively. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of Ofloxacin and Tinidazole bulk drug and in its pharmaceutical dosage form.

Rama Kotaiah.M et al[29] ,(2010) A simple reverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of formoterol fumarate and budesonide in combination. The separation was carried out using a mobile phase consisting of 2mM phosphate buffer and Acetonitrile with pH 3.0 adjusted with phosphoric acid in the ratio of

5%v/v. The column used was Phenomenex C18, (250 mm x 4.6 mm i.d, 5m) with flow rate of 1 ml / min using PDA detection at 303 nm. The described method was linear over a concentration range of 5-50 g/ml and 15-150 g/ml for the assay of Ofloxacin and Tinidazole respectively. Ambroxol (50 g/ml) was used as internal standard. The retention times of Ofloxacin, Tinidazole and Ambroxol were found to be 2.3, 4.1 and 5.1min respectively. Results of analysis were validated statistically and by recovery studies. The limit of quantification (LOQ) for Ofloxacin and Tinidazole were found to be 10 and 30 µg/ml respectively. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of Ofloxacin and Tinidazole bulk drug and in its pharmaceutical dosage form.

Gandhimathi Met al[30],(2006) were developed A simple, accurate and precise high performance thin layer chromatographic for the estimation offormoterolfumarate and budesonide simultaneously in tablet dosage form. The method employed silica gel 60GF254 precoated plates as stationary phase and a mixture of n-butanol: ethanol: ammonia (5:5:4 %v/v/v) as mobile phase. The plate was scanned and quantified at 295 nm using Camag TLC scanner. The method was validated for linearity, accuracy, precision, repeatability and specificity, proving its utility in estimation of ofloxacin and ornidazole in combined dosage form.

Nageswara Rao M et al[31],(2015)were developed A simple, precise and accurate isocratic high-performance liquid chromatography method is developed for the simultaneous estimation of Cefixime and Ornidazole in bulk drug and pharmaceutical dosage form. The separation and quantification is carried out using YMC Pack Pro C18, 250X4.6, 5 um analytical columns. The mobile phase comprises of 0.1M NaHPO: Methanol (500: 500 v/v). The flow rate is 1.2 mL/min

The eluent is monitored at 270 nm. nm. The retention time of cefixime and ornidazole are 3.25min and 4.450 min, respectively. The method is, validated in terms of linerarity, sensitivity , precision. Accuracy, specificity, selectivity and robustness. The stress testing is carried out under acidic, alkaline, oxidation, thermal degradtin and photolytic conditions. The degradation products are well resolved from the cefixime and ornidazole peaks.

Srinivasa Rao K et al[32],(2014)were developed estimation of Ofloxacin (OFL) and Tinidazole (TNZ) in in bulk and pharmaceutical dosage form, without prior separation, by three different techniques (Simultaneous equation, absorbance ratio method and First order derivative method). Materials and Methods: The present work was carried out on Shimadzu electron UV1800 double beam UV-Visible spectrophotometer.The absorption spectra of reference and test solutions were carried out in 1 cm matched quartz cell over the range of 200 - 400 nm. The first method is the application of simultaneous equation. Where the linearity ranges for OFL and TNZ were 5-30 µg/ml and 10-50 µg/ml respectively. The second method is the determination of ratio of absorbance at 278nm, the maximum absorption of TNZ and isobesticwavelength 283 nm, the linearity ranges for OFL and TNZ were 5-30 µg/ml and 10-50µg/ml respectively.

3. OBJECTIVES AND AIM OF THE WORK

3.1. AIM & OBJECTIVE OF THE WORK

There is several basic criteria for new method development of drug molecules. For example a) The drug or drug combination may not be official in any pharmacopoeias b) A proper analytical procedure for the drug may not be available in the literature due to patent regulations. c) The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

It is important for an analyst to develop stable, accurate and validated method for estimation of marketed formulations and specially the widely used formulations. A new combination of Formoterol Fumarate and Budesonide has been selected.

After extensive literature review, The Literature survey reveals that, HPLC and Stability Indicating methods were reported for simultaneous estimation of Formoterol Fumarate with other combinations and HPLC methods are reported for individual drugs but Stability indicating of Formoterol Fumarate and Budesonide combination not reported in any literature.

The present work describes development of simple, precise and accurate, validated stability indicating RP-HPLC method for the simultaneous estimation of formoterol fumerate and budesonide in its pure and pharmaceutical inhalation powder form as per ICH – guidelines.

It becomes necessary, therefore to develop newer analytical method and Stability indicating studies for this drug.

Reported optimized methods are not significantly stable, as per ICH guidelines for stability study maximum cases drugs are degraded, so there is a less justification to indicate the method" stability indicating".

Keeping the above facts in mind the present work was planned to develop such an method which should be sufficiently stable by developed such a mobile phase for analysis, accurate, rapid and validated, by bring down to retention time for both the drug and all validated parameters within limit

3.2. PLAN OF WORK

The number of drugs introduced into the market is increasing every year. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities, development of patient resistance and introduction of better drugs by competitors.

Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

The total work was planned and constructed s follows to achieve the objective of the work Solubility testing of the drugs using different organic solvents.

- Determination of wavelength by using UV- Visible spectrophotometer.
- Checking of linearity by using different concentrations of the drug under study by UV-Visible spectrophotometer method.
- Calibration of HPLC using standard procedures.
- Selection of proper mobile phase the optimization in HPLC using several trials. Setting of proper optimized method as per ICH Q2B guidelines.
- Application of optimized validation method for the analysis of method formulation.
- Perform the stability study as per ICH guidelines.

4. DRUG PROFILE

4.1. FORMOTEROL FUMARATE

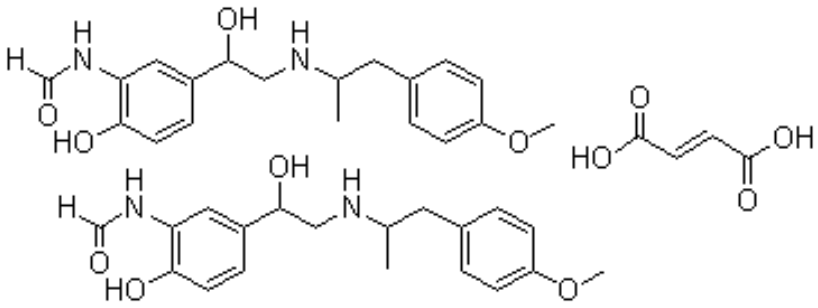

Fig. 3.1Structure of Formoterol Fumarate

Nomenclature:N-[2-hydroxy-5-(1-hydroxy-2-{[1-(4-methoxyphenyl)propan-2-yl]amino}ethyl)phenyl]formamide

Molecular formula : C19H24N2O4

Molecular weight : 344.4049

Solubility: Freely soluble in glacial acetic acid, soluble in methanol, sparingly soluble in ethanol and isopropanol, slightly soluble in water and practically insoluble in acetone, ethyl acetate, and diethyl ether.

Description : Formoterol fumarate is a white to yellowish crystalline powder.

Category: Sympathomimetics, Adrenergic beta-2 Receptor Agonist, Bronchodilators Agents.

4.2. BUDESONIDE

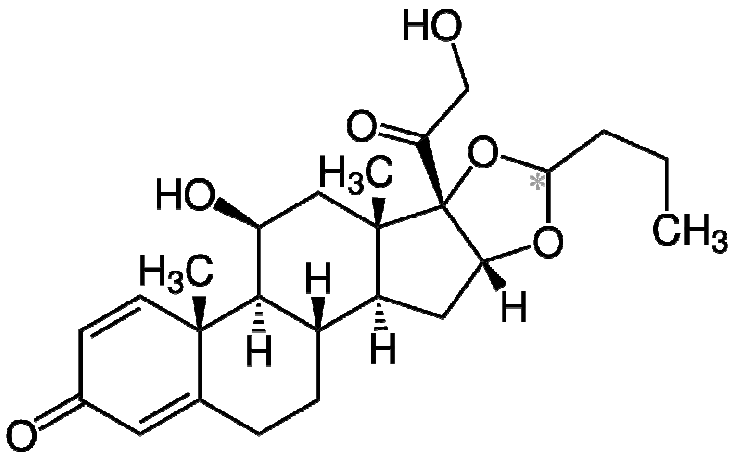

***Fig. 3.2*:** Structure of **Budesonide**

IUPAC Name: (1S,2S,4R,8S,9S,11S,12S,13R)-11-hydroxy-8-(2-hydroxyacetyl)-9,13-dimethyl-6-propyl-5,7dioxapentacyclo[10.8.0.0^{2,9}.0^{4,8}.0^{13,18}]icosa-14,17-dien-16-one

Molecular formula: C25H34O6

Molecular weight: 430.5339

Solubility: Freely Soluble in Chloroform, insoluble in water, heptane,

Sparingly Soluble in ethanol,

Category: Corticosteroids.

5. MATERIALS AND METHODS

5.1. CHEMICALS AND STANDARDS USED

Table 5.1: List of chemicals and standards used

S.No	Chemicals	Manufacturer Name	Grade
1.	Water	Merck	HPLC grade
2.	Methanol	Merck	HPLC grade
3.	Acetonitrile	Merck	HPLC grade
4.	Ortho phosphoric acid	Merck	G.R
5.	KH2PO4	Merck	G.R
6.	K2HPO4	Merck	G.R
7.	0. 22µ membrane filter	Advanced lab	HPLC grade
8.	0.45µ filter paper	Millipore	HPLC grade
9.	FormoterolFumarate and Budesonide	Torrent pharmaceuticals	
10.	FormoterolFumarate and Budesonide (Budamate 400)	Obtained from local pharmacy, Hyderabad	Capsule form

Instruments used

Table 5.2 List of instruments used

S.No	Instrument name	Model number	Soft ware	Manufacturers Name
1	HPLC-auto sampler –PDA detector	Separation module 2690, PDA detector 996	Empower-software version-2	Waters
2	U.V double beam spectrometer	UV 3000+	U.V win soft ware	Lab India
3	Digital weighing balance(sensitivity 5mg)	ER 200A	-	Ascoset
4	pH meter	PHAN	-	Lab India
5	Sonicator	SE60US	-	Enertech

5.2. METHODS

Preparation of various buffers used in trials:

Preparation of Potassium dihydrogen orthophosphate buffer (pH-4.5)

About1.17 g of Potassiumdihydrogen orthophosphate was accurately weighed and taken into 250 ml volumetric flask.

Then add 150 ml of HPLC water,dissolve completely to get a clear solution. Make up the volume up to the mark.

The pH was adjusted to 4.5 with Orthophosphoric acid and filtered through 0.45 µm membrane filter Sonicate for 15 min.

Preparation of mobile phase:

A Combination of Potassium dihydrogen orthophosphate buffer (pH-4.5)(*refer 7.2.1.1*)and Acetonitrile was mixed in the ratio of 30:70, The pH was adjusted to 4.5 with Orthophosphoric acid and filtered through 0.45 µm membrane filter.

This prepared solution was used as mobile phase. This solution was also used for specificity blank solution.

Preparation of standard solution of Formoterol Fumarate and Budesonide for trials:

Standard solution of Formoterol Fumarate and Budesonide were prepared by dissolving 10 mg of each drug in 10 mL of mobile phase. Further dilution was made by adding 1 mL of the stock solution to 10 mL standard flask and making up the volume with the mobile phase.

Preparation of Solutions for assay

Preparation of the Formoterol Fumarate and Budesonide standard and sample solution

Sample solution preparation

22 mg of Formoterol Fumarate and Budesonide capsule powder were accurately weighed and transferred into a 10 ml clean dry volumetric flask, 2 ml of diluent was added and sonicate to dissolve it completely and making volume up to the

mark with the same solvent(Stock solution). Further pipette out 0.2 ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

Standard solution preparation

10 mg Formoterol Fumarate and 15 mg of Budesonide working standard was accurately weighed and transferred into a 10ml clean dry volumetric flasks separately and add about 2 ml of diluent to both flasks and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 0.3ml and 0.45ml of the above stock solutions into a 10ml volumetric flask and was diluted up to the mark with diluent.

6. RESULTS AND DISCUSSION

6.1. SOLUBILITY STUDIES

These studies are carried out at 25 °C

Table 6.1: Solubility studies

Solvent Name	FormoterolFumarate	Budesonide
Water	Soluble	Insoluble
Methanol	Freely Soluble	Freely Soluble
Acetonitrile	Soluble	Soluble

6.2. DETERMINATION OF WORKING WAVELENGTH (λmax)

In simultaneous estimation of two drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate both drugs accurately.

6.2.1. Preparation of standard solution of Formoterol Fumarate

About 50mg of Formoterol Fumarate was weighed and transferred in to 50ml volumetric flask and dissolved in 25mL of methanol and then make up to the mark with the mobile phase and 10µg /mL of solution was prepared by diluting 0.1mL to 10mL with mobile phase

6.2.2. Preparation of standard solution of Budesonide

About 50mg of Budesonide was weighed and transferred into 50ml volumetric flask and dissolved in 25mL of mobile phase and then diluted up to the mark with mobile phase and 10µg /mL of solution was prepared by diluting 0.1mL to 10mL with mobile phase.

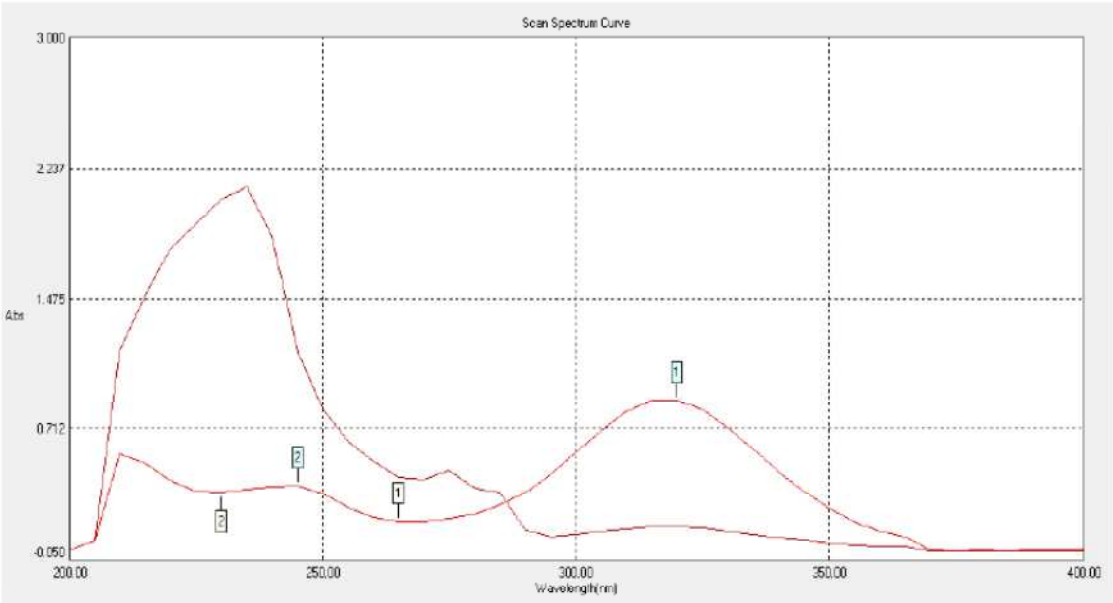

Fig.6.1: Selection of wavelength

6.2.3. Results

The wavelength at 228 nm has been selected, as the isobestic point.

6.3. METHOD DEVELOPMENT

Aliquots of the mixed solutions containing FormoterolFumarateand Budesonide were prepared and a number of eluting experiments were conducted for the optimization of separation of drugs using mobile phase.

Trial - 1

For Formoterol Fumarate

Mobile Phase: Water and Methanol were mixed in the ratio of 30:70 and sonicated to degas.20 µL of prepared solution was injected into the HPLC and the chromatograms were recorded

Chromatographic conditions

Name of the column	:	waterssymmetryC18 (4.6*150mm) 5µm
Mobile phase	:	Water : Methanol (30:70)
Elution mode	:	Isocratic.
Flow rate	:	1 mL/min.
Detection wavelength	:	228 nm.
Injection volume	:	20.00 µL.
Run time	:	10.00 min.
Retention Time	:	3.662 min.

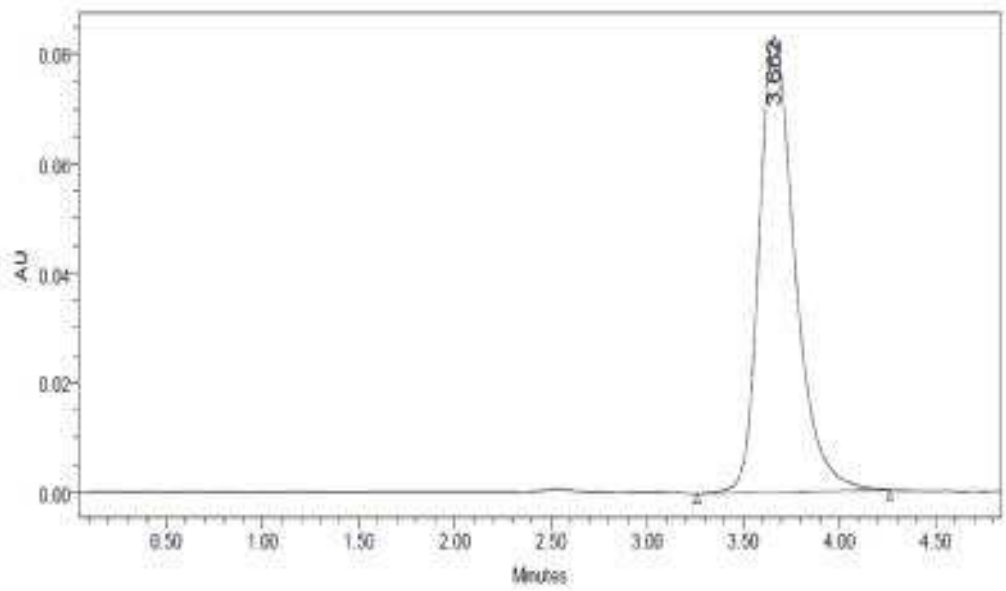

Fig. 6.2**:**Chromatogram of Trial – 1

Table 6.2: Results of Trial -1

Drugs	**RT**	**Peak Area**	**TP**	**TF**	**Rs**
FF	3.662	83319	2975	1.35	-

Observation & Conclusion:

Formoterol Fumarate is eluted at 3.662 min and the results of the chromatogram along with peak area as shown as below and the results of the chromatogram are given in Table 6.2. From the chromatogram it was observed that the retention time, theoretical plates and tailing factor for FF was good. So no more trials were required for obtaining proper retention time, go for next trail for Budesonide.

***Trial– 2*:- For Budesonide**

Mobile Phase :

Water and Methanol were mixed in the ratio of 20:80 andsonicated to degas.20 µLof prepared solution was injected into the HPLC and the chromatograms were recorded

Chromatographic conditions

Name of the column	:	waterssymmetryC18 (4.6 χ 150mm) 5µm
Mobile phase	:	Water : Methanol (20:80)
Elution mode	:	Isocratic.
Flow rate	:	1 mL/min.
Detection wavelength	:	228 nm.
Injection volume	:	20.00 µL.
Run time	:	10.00 min.
Retention Time	:	1.514 min.

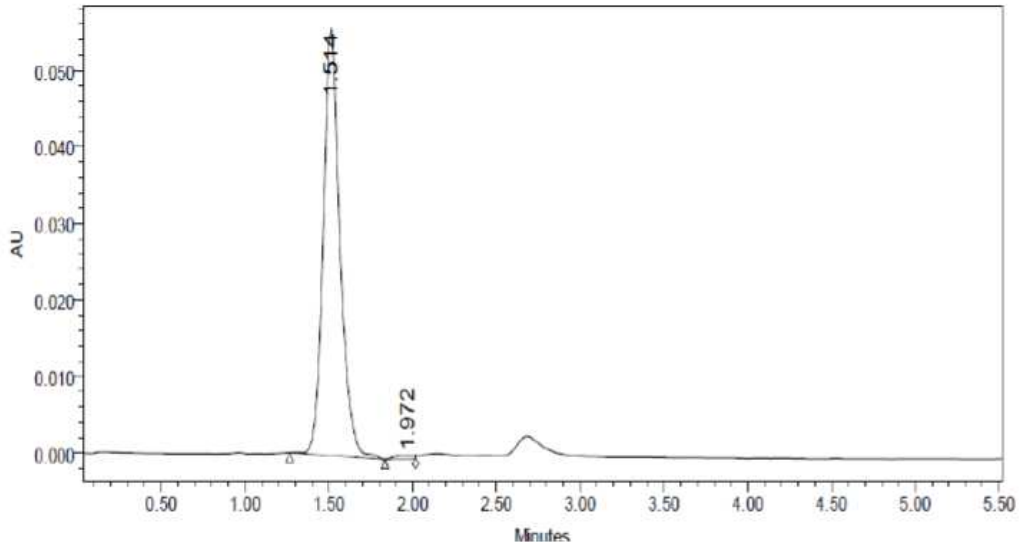

Fig. 6.3**:** Chromatogram of Trial – 2

\Table 6.2: Results of Trial -2

Drugs	**RT**	**Peak Area**	**TP**	**TF**	**Rs**
BU	1.514	388193	1162	1.19	-

Observation& Conclusion:

Budesonide is eluted at 1.514 min and the chromatogram along with peak area as shown as above. Theoretical plates, tailing factor and the results of the chromatogram are given table 6.2 .From the chromatogram it is observed that theoretical plates were not good, so go for next trial.

Trial – 3

Mobile Phase:

Water and Methanol were mixed in the ratio of 30:70 and sonicated to degas.20 µL of prepared solution was injected into the HPLC and the chromatograms were recorded

Chromatographic conditions

Name of the column	:	Waters symmetry C18 (150x4.6 ID) 3.5 µm
Mobile phase	:	Water : Methanol (30:70)
Elution mode	:	Isocratic.
Flow rate	:	1 mL/min.
Detection wavelength :		268 nm
Injection volume	:	20.00 µL.
Run time	:	8.00 min.
Retention Time	:	2.422 min.

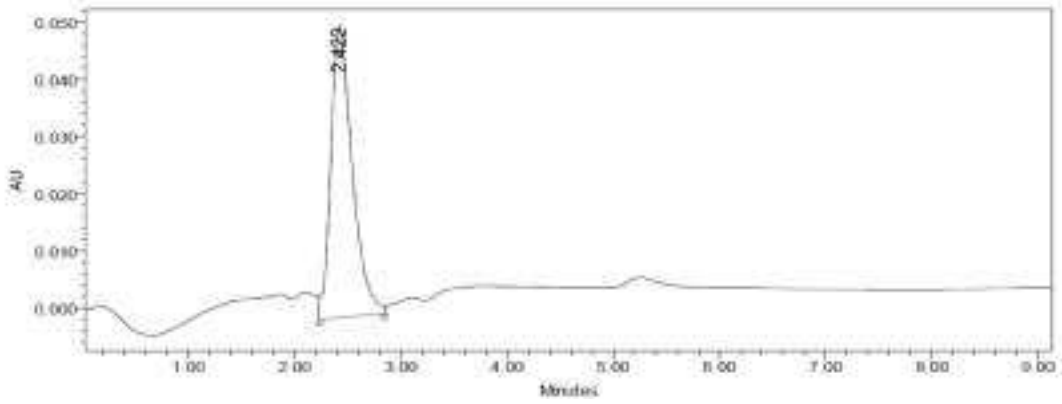

Fig. 6.4: Chromatogram of Trial –3.

Table 6.4: Results of Trial -2

Drugs	**RT**	**Peak Area**	**TP**	**TF**	**Rs**
BU	2.422	713281	1720	1.17	-

Observation & Conclusion:

Budesonide is eluted at 2.422 min and the chromatogram along with peak area as shown. . From the chromatogram it is observed retention time was good. So go for combination.

***Trial -4* :- Combination of both drugs**

Mobile Phase:

Potassium dihydrogen phosphate buffer (pH-4.5), Methanol were mixed in the ratio of 20 : 80.It was filtered through 0.45 µm membrane filter and degassed, 20 µLof prepared solution was injected into the HPLC and the chromatograms were recorded.

Chromatographic conditions

Name of the column : Waters symmetry C18 (150x4.6 ID) 3.5 µm
Mobile phase : Potassium dihydrogen phosphate buffer
pH - 4.5) :Methanol (20:80)
Elution mode : Isocratic.
Flow rate : 1 mL/min.
Detection wavelength : 228 nm.
Injection volume : 20.00 µL.
Run time : 10.00 min.
Retention Time : FF - 1.593 min & BU - 3.526 min.

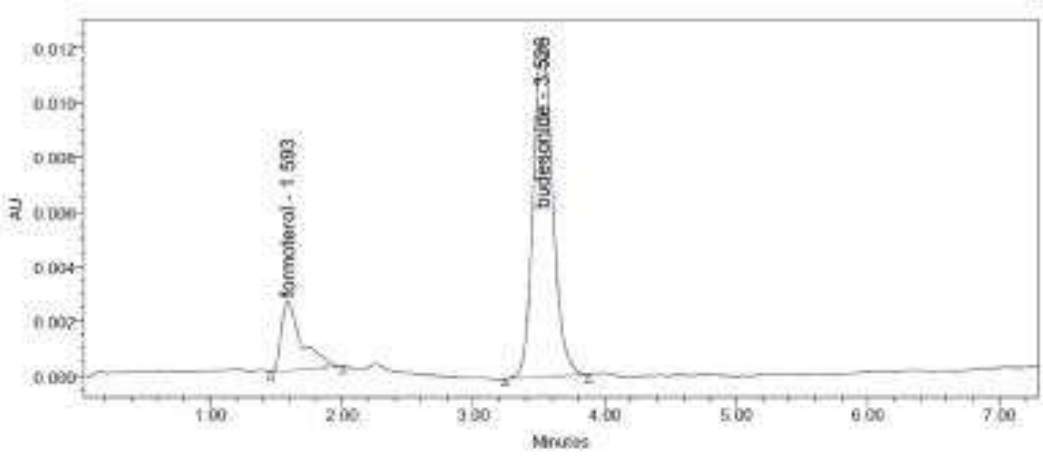

Fig. 6.5**:**Chromatogram of Trial – 4

Table 6.5:Results of Trial -4

Drug	RT	Peak Area	TP	TF	Rs
FF	1.593	27221	2760	2.05	-
BU	3.526	126318	2813	1.16	7.7

Observation & Conclusion*:*

From the figure 8.4it was observed that FF is eluted at 1.593 min and BU is eluted at 3.526 min. The results of the chromatogram along with peak area, theoretical plates, tailing factor and resolution were given above. From the chromatogram it was observed that the sample solution injected consisted of two drugs and separation was not good and tailing factor for FF is not within limits. Therefore this trial was not considered.

Trial – 5

Mobile Phase:

Potassium dihydrogen phosphate buffer (pH-4.5),Methanol were mixed in the ratio of 30 : 70.It was filtered through 0.45 µm membrane filter and degassed20 µLof prepared solution was injected into the HPLC and the chromatograms were recorded.

Chromatographic conditions

Name of the column	:	Waters symmetry C18 (150x4.6 ID) 3.5 µm
Mobile phase	:	Potassium dihydrogen phosphate buffer
pH - 4.5) :Methanol (30:70)		
Elution mode	:	Isocratic.
Flow rate	:	1 mL/min.
Detection wavelength :		228 nm.
Injection volume	:	20.00 µL.
Run time	:	10.00 min.
Retention Time	:	FF - 1.985 min. BU - 2.709 min.

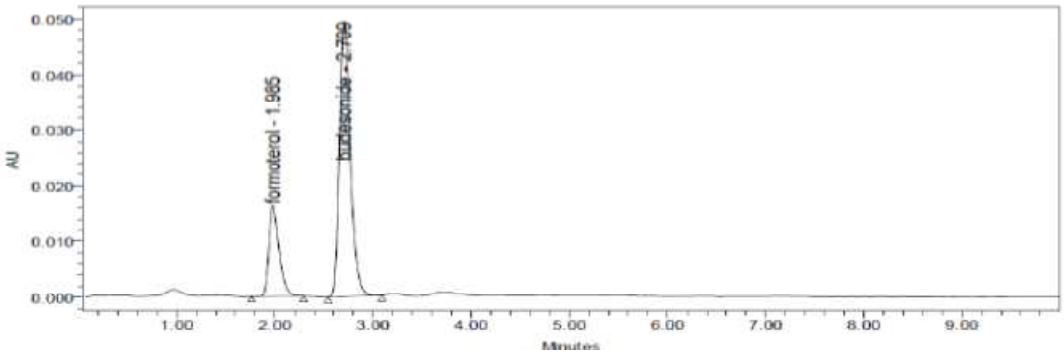

Fig. 6.6:Chromatogram of Trial -5

Table 6.6:Results of Trial -5

Drug	RT	Peak Area	TP	TF	Rs
FF	1.985	117004	1728	1.38	-
BU	2.709	382091	2779	1.29	3.6

Observation& Conclusion:

From the figure 6.6 it is observed that FF is eluted at 1.985 min and BU is eluted at 2.709 min. The results of the chromatogram along with peak area, theoretical plates, tailing factor and resolution are given. From the chromatogram it was observed that the sample solution injected consisted of two drugs and separation was not good and retention time and theoretical plates for FF is not good. Therefore this trial was not considered

Trial - 6

Mobile Phase:

Potassium dihydrogen phosphate buffer (pH-4.5), Acetonitrile were mixed in the ratio of 20: 80.It was filtered through 0.45 µm membrane filter and degassed20 µL of prepared solution was injected into the HPLC and the chromatograms were recorded.

Chromatographic conditions

Name of the column	:	Waters symmetry C18 (150x4.6 ID) 3.5 µm
Mobile phase	:	Potassium dihydrogen phosphate buffer
pH - 4.5) :Acetonitrile (20:80)		
Elution mode	:	Isocratic.
Flow rate	:	1 mL/min.
Detection wavelength	:	228 nm.
Injection volume	:	20.00 µL.
Run time	:	10.00 min.
Retention Time	:	FF - 1.782 min.BU - 4.006 min.

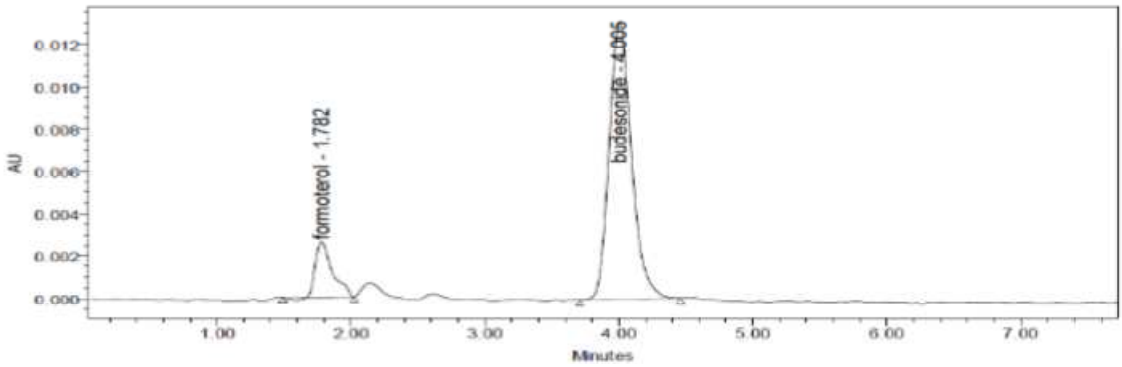

Fig. 6.7:Chromatogram of Trial -6

Table 6.7:Results of *Trial -6*

Drug	RT	Peak Area	TP	TF	Rs
FF	1.782	24154	3164	1.61	-
BU	4.006	13125	2892	1.21	8.4

Observation & Conclusion:

Though the resolution and tailing factor were good for FF and BU, the retention time was not satisfactory for FF and further trials has been done in order to achieve good retention time.

Trial –7

Mobile phase

Potassium dihydrogen phosphate buffer (pH-4.5), Acetonitrile were mixed in the ratio of 25 : 75.It was filtered through 0.45 µm membrane filter and degassed20 µL of prepared solution was injected into the HPLC and the chromatograms were recorded.

Chromatographic conditions

Name of the column	:	Waters symmetry C18 (150x4.6 ID) 3.5 µm
Mobile phase	:	Potassium dihydrogen phosphate buffer
pH - 4.5) :Acetonitrile (25:75)		
Elution mode	:	Isocratic.
Flow rate	:	1 mL/min.
Detection wavelength :		228 nm.
Injection volume	:	20.00 µL.
Run time	:	10.00 min.
Retention Time	:	FF - 2.034 min. BU - 3.511 min.

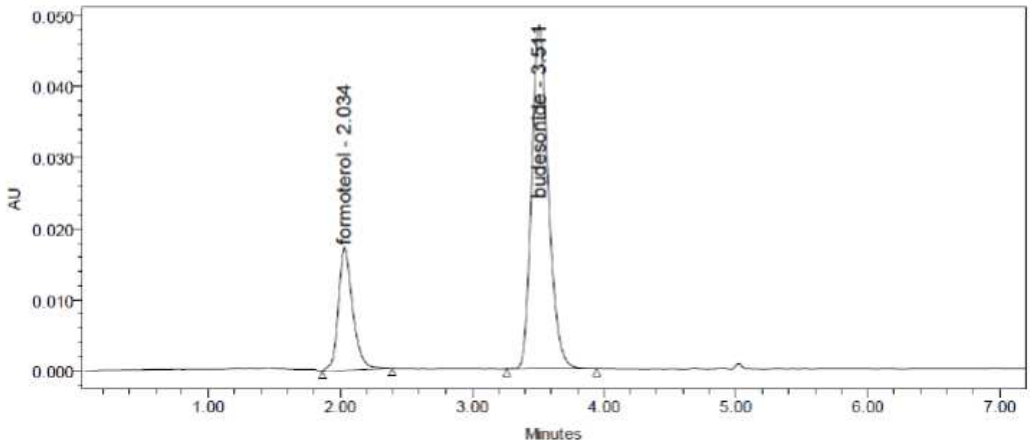

Fig. 6.8 Chromatogram of Trial -7

Table 6.8: Results of Trial -6

Drug	RT	Peak Area	TP	TF	Rs
FF	2.034	133805	1708	1.19	-
BU	3.511	423920	3721	1.21	6.7

Observation & Conclusion:

The FF peak was observed at 2.034min, BU peak was observed at 3.511 min with tailing factors 1.19 and 1.21 respectively. Lack of enough theoretical plates was observed for FF. Hence this trial was not considered. So go for next trails.

Trial-8

Mobile phase

Potassium dihydrogen phosphate buffer (pH-4.5), Acetonitrile were mixed in the ratio of 30: 70.It was filtered through 0.45 µm membrane filter and degassed20 µLof prepared solution was injected into the HPLC and the chromatograms were recorded.

Chromatographic conditions

Name of the column : Waters symmetry C18 (150x4.6 ID) 3.5 µm

Mobile phase : Potassium dihydrogen phosphate buffer

pH - 4.5) :Acetonitrile (30:70)

Elution mode : Isocratic.

Flow rate : 1 mL/min.

Detection wavelength : 228 nm.

Injection volume : 20.00 µL.

Run time : 10.00 min.

Retention Time : FF - 2.051 min.& BU - 4.234 min

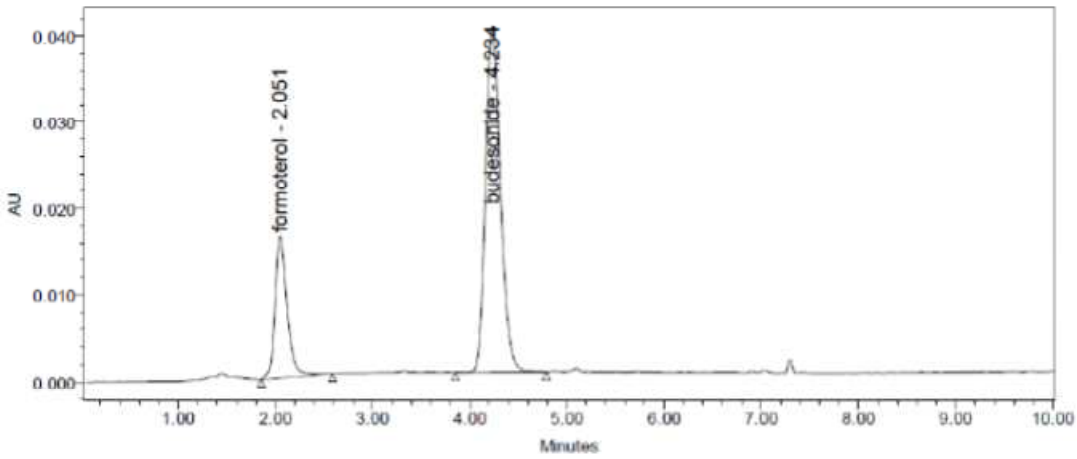

Fig. 6.9: Chromatogram of Trial -8

Table6.9:Results of Trial -8

Drug	RT	Peak Area	TP	TF	Rs
FF	2.051	140672	2419	1.17	-
BU	4.234	427794	3591	1.21	8.6

Observation& Conclusion:

The retention time was good for both FF and BU, the tailing factor was less than 2 and the number of theoretical plates were more than 2000 for both and the resolution was good, the details are given So this trail has been chosen as optimised method and further work was continued with this developed method for the validation.

6.4.Optimised chromatographic conditions

Table 6.10: showing chromatographic conditions of the optimized parameters

Parameters	Conditions
Stationary phase (column)	Waters symmetry C18 (150x4.6 ID) 3.5 µm)
Mobile phase	Potassium dihydrogen phosphate buffer pH - 4.5) :Acetonitrile (30:70)
Flow rate (mL/min)	1.0 mL/min
Run time (minutes)	10 min
Column temperature (°C)	Room temperature(20-25^{O}C)
Volume of injection loop (µL)	20µL
Detection wavelength (nm)	228 nm

Table 6.11*:* Results of FF and BU (Sample) for assay

ASSAY

Name	Retention time (min)	Peak Area	TF	Efficiency	Resolution
FF	2.093	120404	1.35	2729.22	-
BU	4.361	426416	1.19	4421.79	9.54

- Preparation of samples for Assay
- Preparation of Standard stock solution
- Preparation of working standard solution
- Preparation of Test stock solution
- Preparation of working standard solution

Calculation

The amount of Budesonide and Formoterol Fumarate present in the formulation by using the formula given below, and results shown in a below table:

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

Where,

AS: Average peak area due to standard preparation

AT: Peak area due to assay preparation

WS: Weight of Budesonide / Formoterol Fumarate in mg

WT: Weight of sample in assay preparation

DT: Dilution of assay preparation

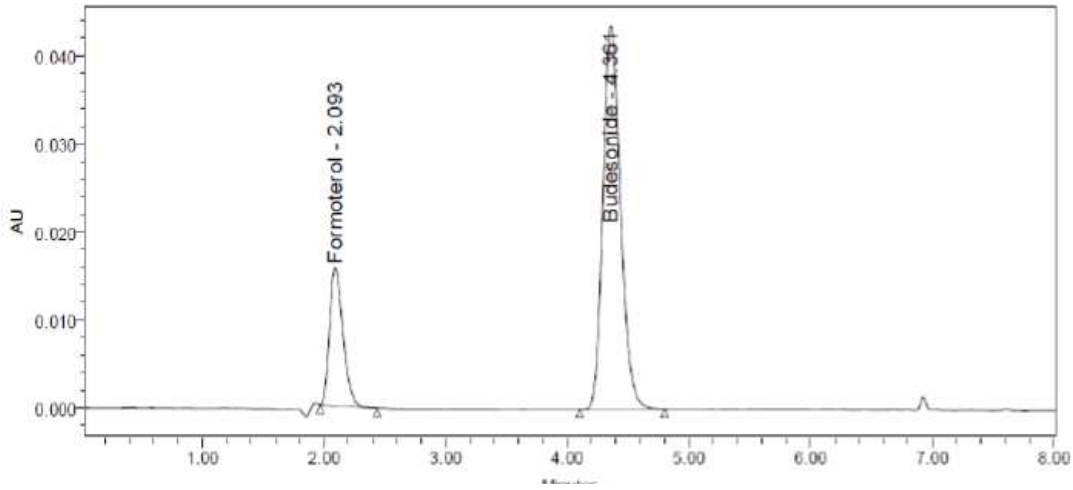

Fig.6.10: Chromatogram of sample solution for Assay

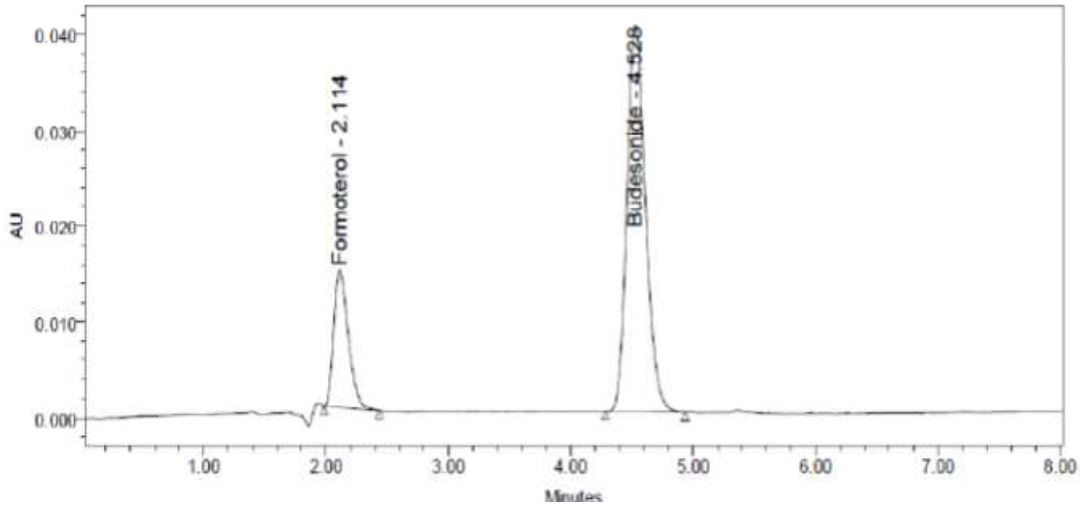

Fig.6 11: Chromatogram of standard solution for Assay

Table 6.12: Results of FF and BU (Standard) for Assay

Drug	**RT (min)**	**Peak Area**	**TF**	**Efficiency**	**Resolution**
FF	2.114	118059	1.40	3502.30	-
BU	4.528	424907	1.20	4222.64	9.53

Table 6.13*:* Results of assay from tablet dosage form

Drug	**Label claim(mg)**	**Amount found(mg)**	**% Assay**
FF	0.006	0.0058	98.05
BU	0.4	0.3986	99.65

Observation:

From chromatograms of sample solution , it was observed that the sample peaks found to be without any interference and% Assay of FF was found to be 98.05 % and Assay of BU was found to be 99.65 % .It was concluded that the % Assay was within the limits (98 % - 102 %).

6.5. VALIDATION OF HPLC FOR METHOD DEVELOPMENT

6.5.1. System suitability

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters like theoretical plates, resolution and asymmetric factor were evaluated.

Table 6.14: Results for system suitability of FormoterolFumarate

Injection	RT(min)	Peak area	TP	TR
1	2.095	127651	1564.31	1.29
2	2.095	127376	1634.55	1.31
3	2.095	128904	1563.37	1.31
4	2.096	126372	1612.78	1.29
5	2.096	128143	1560.59	1.31
6	2.098	127305	1624.48	1.29
Mean		127625	--	--
SD		853.0	--	--
%RSD		0.7	--	--

Table 6.15:Results for system suitability of Budesonide

Injection	RT(min)	Peak area	TP	TF
1	4.337	434309	4325.32	1.18
2	4.344	436839	4242.75	1.18
3	4.346	436814	4282.56	1.18
4	4.348	435350	4384.18	1.18
5	4.353	435462	4322.24	1.18
6	4.354	439095	4348.18	1.19
Mean		436311.4	-	-
SD		1669.5	-	-
%RSD		0.4	-	-

Acceptance criteria

The % RSD for the retention times of Formoterol Fumarateand Budesonide Peaks from 6 replicate injections of each Standard solution should be not more than 2.0 %
The number of theoretical plates (N) for the Formoterol Fumarate and Budesonide peaks is not less than 2000.

The Tailing factor (T) for the Formoterol Fumarate and Budesonide peak is not more than 2.0.

Observation:

The % RSD for the retention times and peak area of Formoterol Fumarate and Budesonide were found to be less than 2%. The plate count and tailing factor results were found to be satisfactory and are found to be within the limit.

6.6 Specificity

Preparation of blank solution. The Mobile phase, Potassium dihydrogen phosphate buffer pH - 4.5) :Acetonitrile (30:70) was taken as blank solution.

Preparation of standard solution Tablet sample preparation

The above prepared solutions were injected and the chromatograms were recorded for the same. The chromatogram for blank is shown figure 6.12.The chromatogram for the standard solution is given in figure 6.14 and the results of the chromatogram are given the chromatogram for the test sample i.e tablet sample is given and the results of the chromatogram are given in figure 6.13.

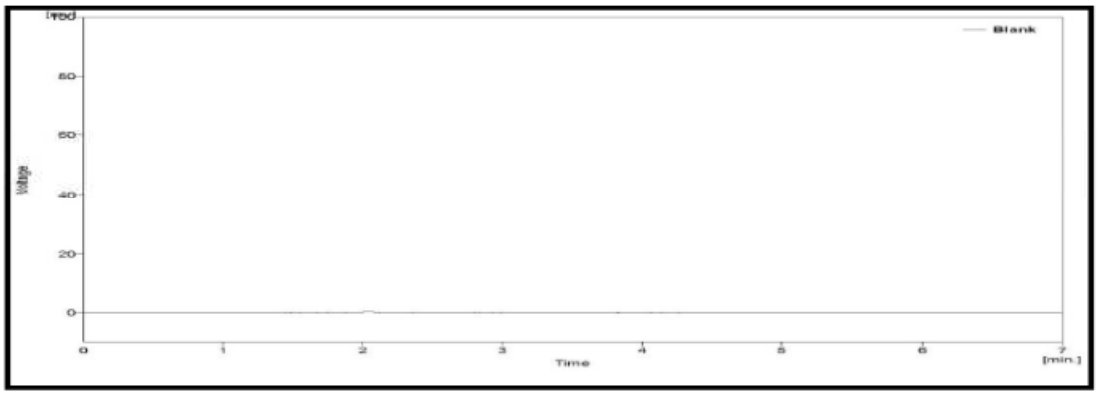

Fig. 6.12: Chromatogram of blank

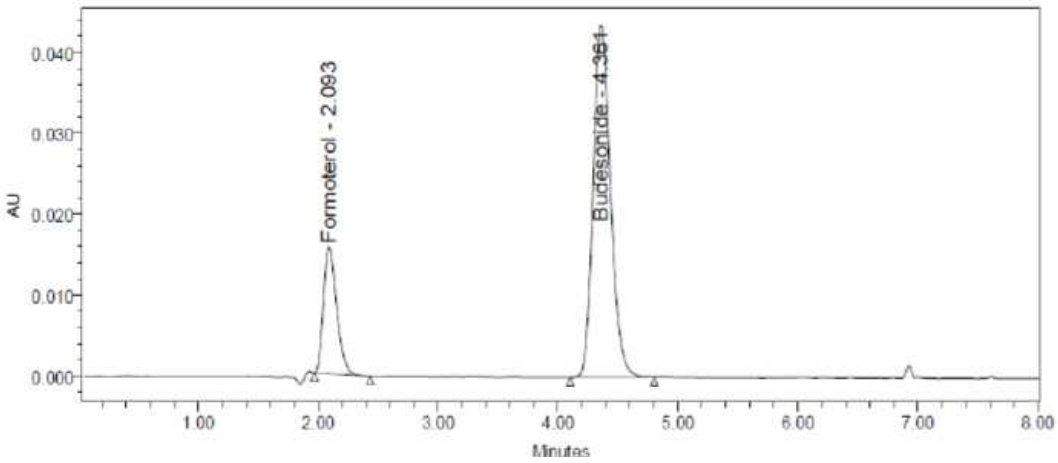

Fig. 6.13*:*Chromatogram for specificity of FF and BU sample

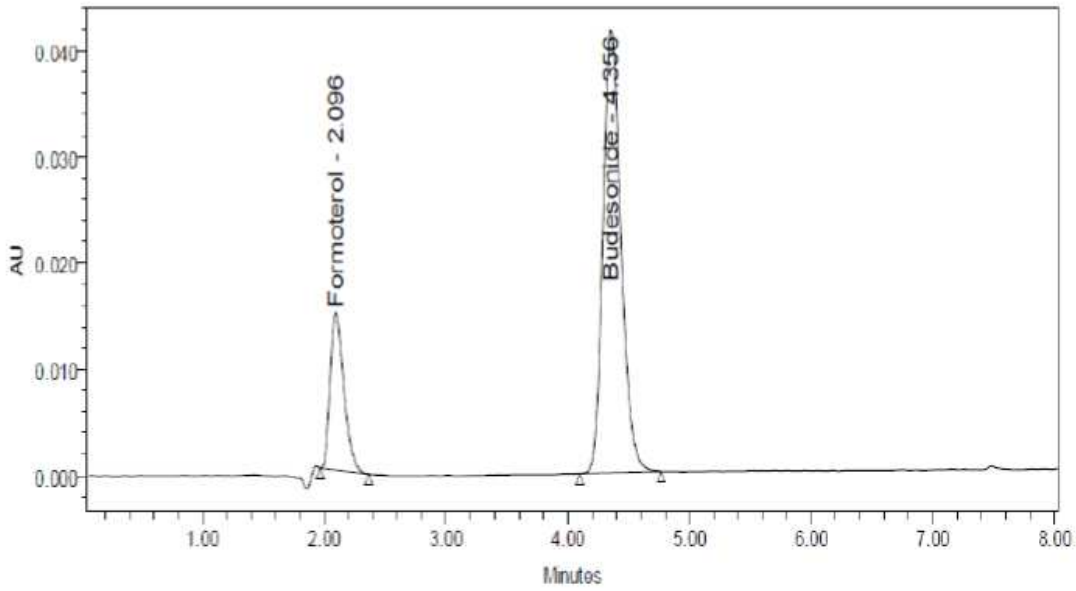

Fig. 6.14:Chromatogram for Specificity of FF and BUstandard

Table.6.16: For Specificity of FormoterolFumarate and Budesonide sample

Drug	RT(min)	Peak Area	TF	Efficiency	Resolution
FF	2.093	120404	1.35	2729	-
BU	4.361	436416	1.19	4422	9.5

Table 6.17: Specificity of FormoterolFumarate and Budesonide standard

Drug	Retention time (min)	Peak Area	TF	Efficiency	Resolution
FF	2.096	116063	1.40	2572	-
BU	4.356	428498	1.21	4211	9.2

Observation:

It was observed from the above data, diluent or excipient peaks are not interfering with the Formoterol Fumarate and Budesonide peaks.

❖ Linearity and range

Preparation of standard stock solution

Preparation of working standard solution

The working standard solution was prepared from the standard stock solution .The prepared working standard solutions were injected and the chromatograms were recorded for the same as shown in figures.6.15-6.

From the obtained data, a graph between the concentration of the drug and peak area are plotted based on data given in figure .for Formoterol Fumarate and Budesonide respectively and the linearity graphs are given below

Table.6.18: Preparations for Linearity

Preparations	Volume from Standard stock transferred in ml	Volume made up in ml (with diluent)	Concentration of solution(µg /ml)	
			FORMO	BUDESO
Preparation 1	0.1	10	10	15
Preparation 2	0.2	10	20	30
Preparation 3	0.3	10	30	45
Preparation 4	0.4	10	40	60
Preparation 5	0.5	10	50	75

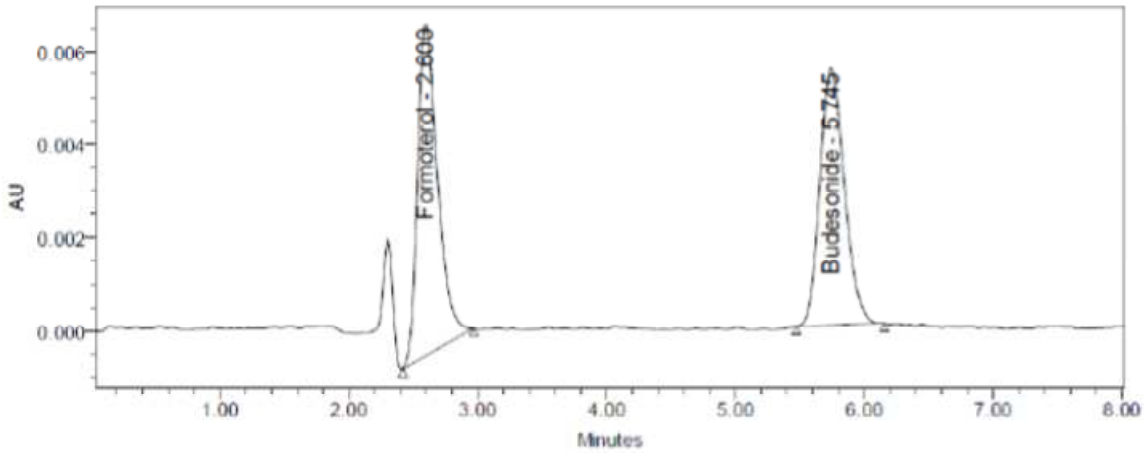

Fig. 6.15*:* Chromatogram of Formoterol Fumarate and Budesonide preparation 1

Table: 6.19: Results of preparation-1 for Linearity

Drugss	**RT (min)**	**Peak Area**	**TF**	**Efficiency**	**Resolution**
FF	2.600	76879	1.29	2353.34	-
B	5.745	72549	1.18	4293.09	9.75

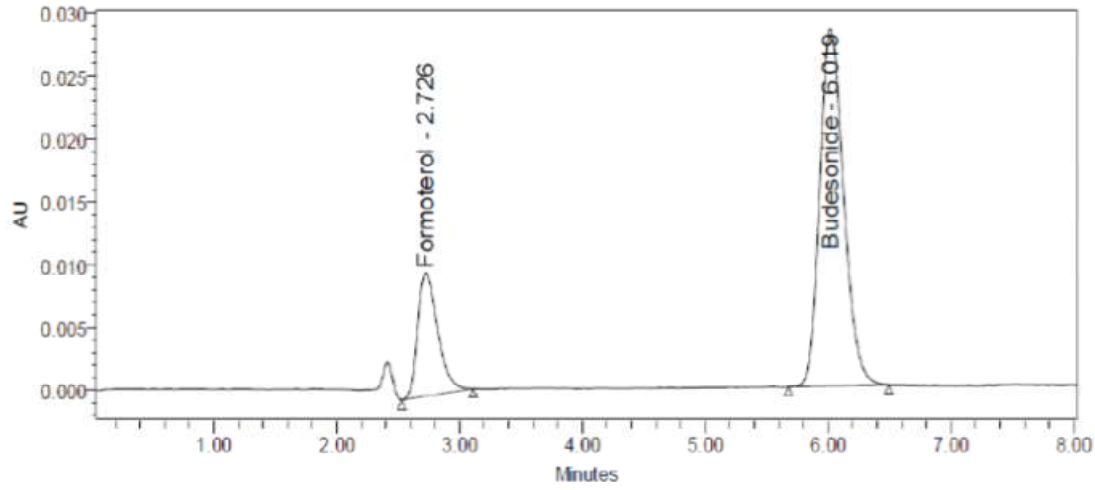

Fig. 6.16: Chromatogram of FormoterolFumarate and Budesonide preparation-2

Table 6.20: Results of preparation-2 for Linearity

Drugs	**Retention time (min)**	**Area**	**TF**	**Efficiency**	**Resolution**
FF	2.690	174699	1.36	2463.36	-
BU	5.891	578153	1.21	4398.06	9.89

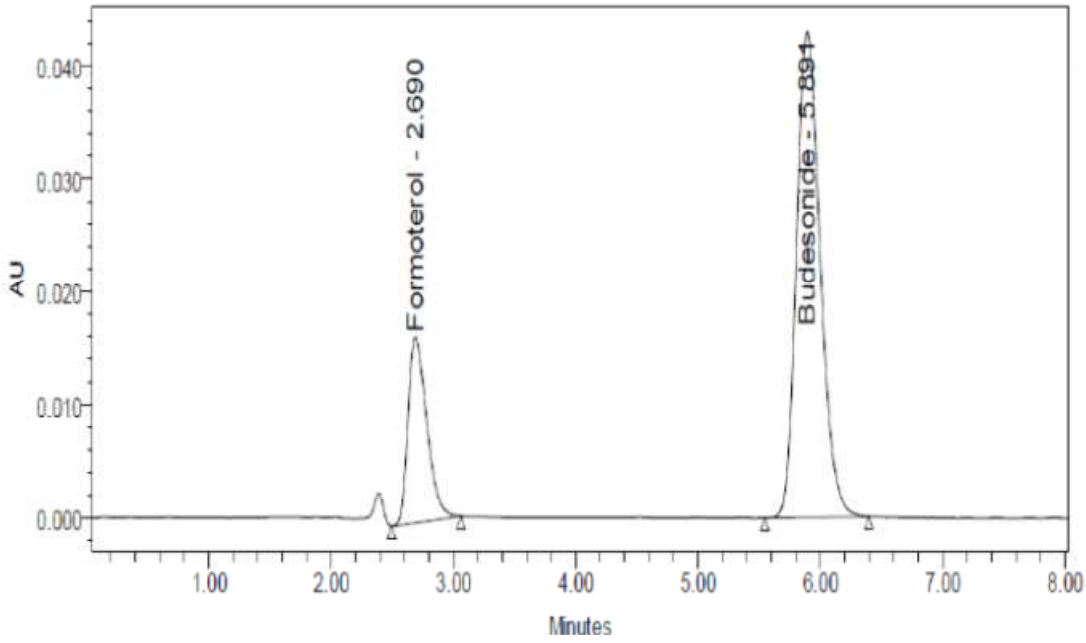

Fig. 6.17:ChromatogramFormoterolFumarate and Budesonide preparation-3

Table 6.21*:* Results of preparation-3 for Linearity

Drug	RT (min)	Peak Area	TF	Efficiency	Resolution
FF	2.726	110214	1.33	2376.70	-
BU	6.019	384068	1.21	4557.69	9.94

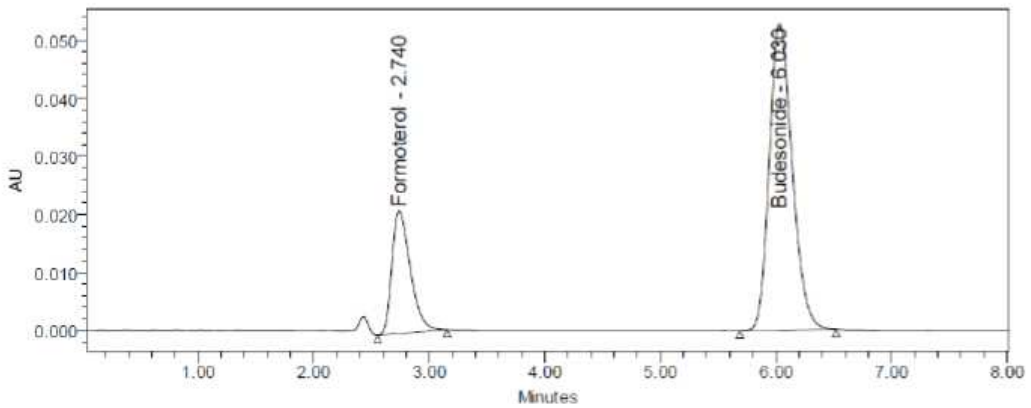

Fig. 6.18:Chromatogram of FormoterolFumarate and Budesonide preparation-4

Table. 6.22:Results of preparation-4 for Linearity

Drug	RT(min)	Area	TF	Efficiency	Resolution
FF	2.740	233585	1.39	2440.18	-
BU	6.030	726703	1.21	4407.77	9.86

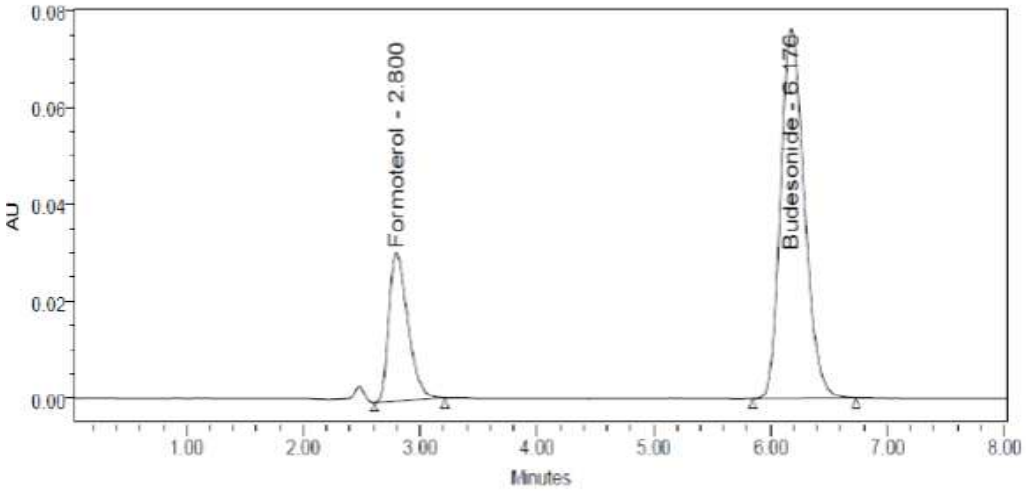

Fig. 6.19:Chromatogram Formoterol Fumarate and Budesonide preparation-5

Table 6.23: Linearity data for preparation 5

Drug	RT (min)	Peak Area	TF	Efficiency	R esolution
FF	2.800	344428	1.40	2434.75	-
BU	6.176	1075106	1.22	4392.20	9.90

Table 6.24*:* Results Linearity data of Formoterol Fumarate and Budesonide

S.NO.	Conc(µg)	Area	Conc(µg)	Area
1.	15	76879	10	72549
2.	30	174699	20	578153
3.	45	110214	30	384068
4.	60	233585	40	726703
5.	75	344428	50	1075106

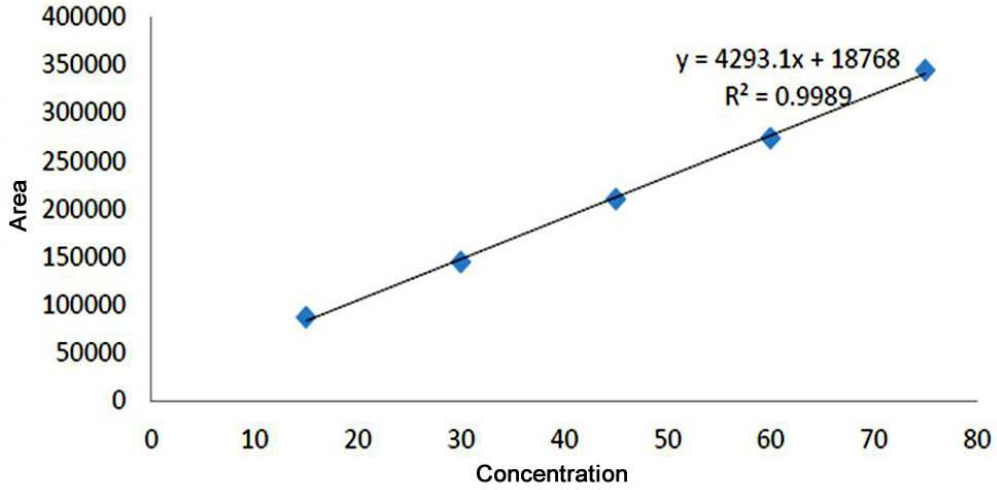

Fig.6.20*:*Linearity graph of Formoterol Fumarate

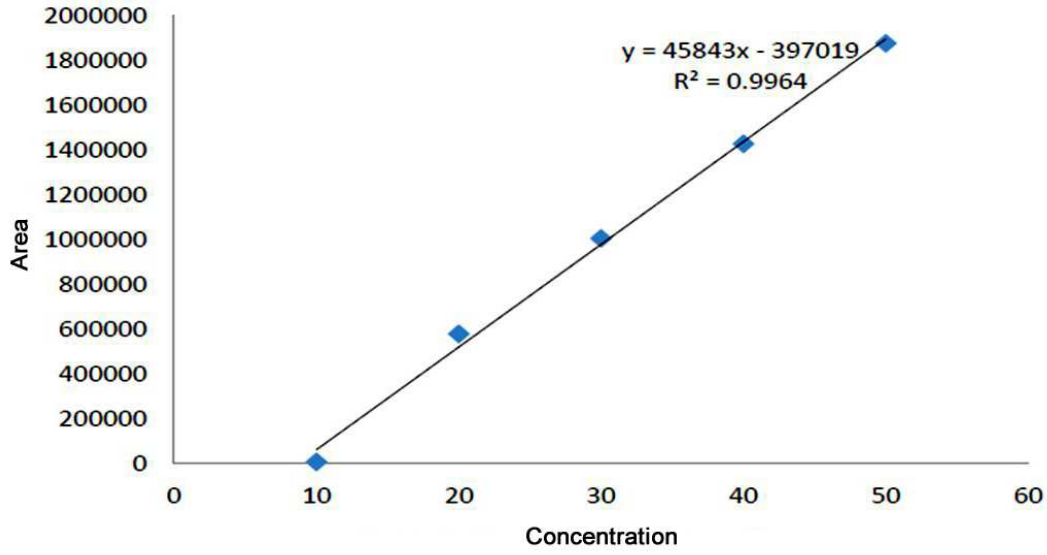

Fig.6.21*:* Linearity graph of Budesonide

Acceptance criteria

The relationship between the concentration (in %) of Formoterol Fumarate and Budesonide and area of Formoterol Fumarate and Budesonide should be linear in the specified range and the correlation should not be less than 0.99.

Observation

The correlation coefficient for linear curve obtained between concentration vs. Area for standard preparations Formoterol Fumarate and Budesonide is 0.998 and 0.996. The relationship between the concentration of Formoterol Fumarate and Budesonide and area of Formoterol Fumarate and Budesonide is linear in the range examined since all points lie in a straight line and the correlation coefficient is well within limits.

❖ Accuracy

Accuracy of the method was determined by Recovery studies. To the formulation (pre-analysed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 50%, 100%, 150%.

Preparation of standard stock solution (spikingPreparation of Test stock solution

Preparation of sample solutions

A. **For preparation of 50% solution (with respect to target assay concentration)**

5 mg of budesonide and 5 mg of Formoterol working standard were accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with The same solvent (Stock Solution).Further pipette out 1 ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

B. For preparation of 100% solution (with respect to target assay concentration)

10 mg of budesonide and 10 mg of formoterolworking standards were accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 1 ml of above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

C. For preparation of 150% solution (with respect to target assay concentration)

15 mg of budesonide and 15 mg of formoterol working standards into a 10ml clean dry volumetric flask add about 2 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipetteout 1 ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

Procedure

The standard solutions of accuracy 50%, 100% and 150%were injected into chromatographic system. Calculate the amount found and amount added for budesonide and Formoterol. Calculated the individual % recovery and mean % recovery values of each.

Procedure

The standard solutions of accuracy 50%, 100% and 150%were injected into chromatographic system. Calculate the amount found and amount added for budesonide and formoteroland calculate the individual % recovery and mean % recovery values.

For 50 % Recovery

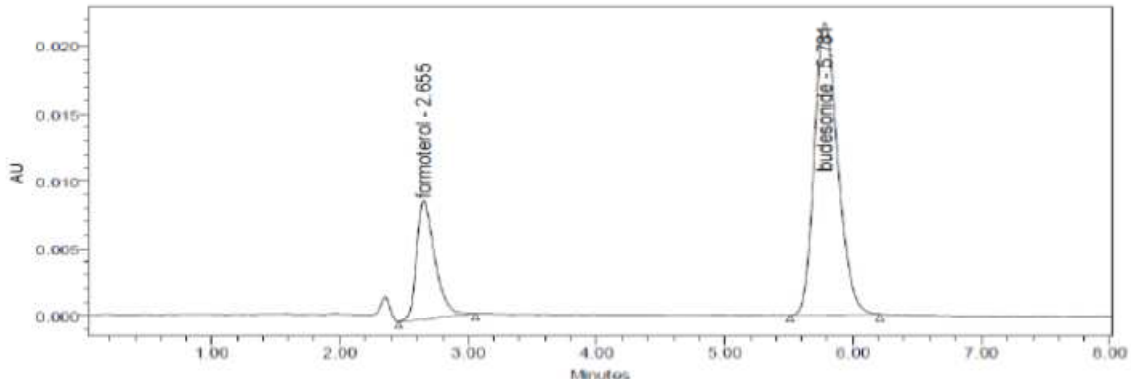

Fig. 6.22: Chromatogram of 50% recovery – injection 1

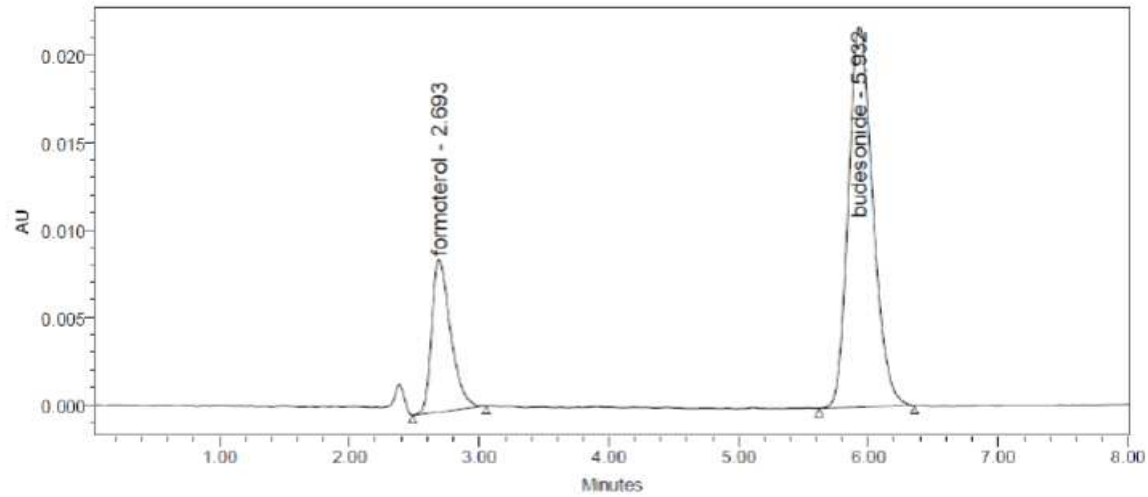

Fig. 6.23: Chromatogram of 50% recovery – injection 2

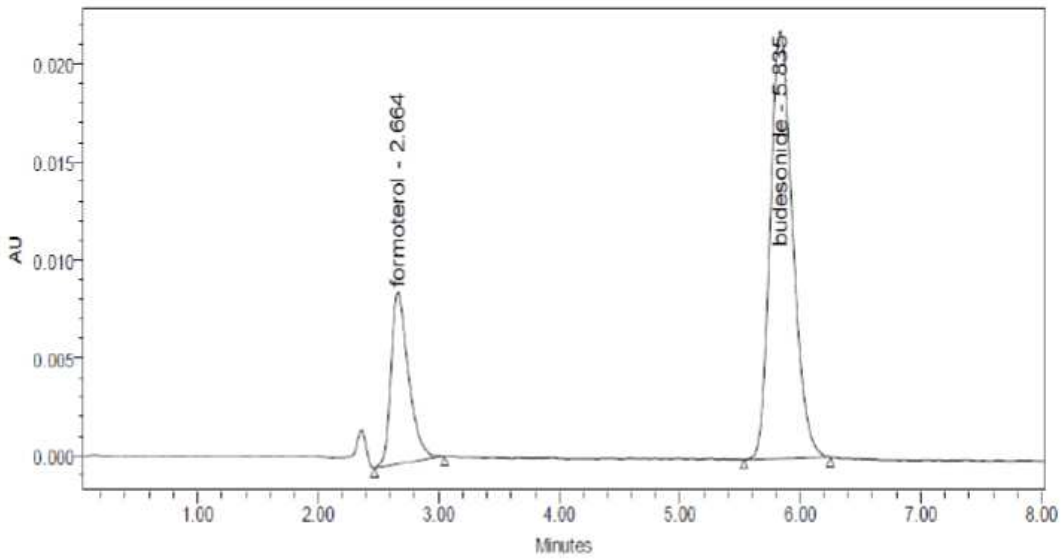

Fig.6.24: Chromatogram of 50% recovery – injection 3

Table 6.25:Results for 50% Recovery

Injection	FF		BU	
	RT	Area	RT	Area
1	2.655	87403	5.781	278081
2	2.693	88028	5.932	285613
3	2.664	88863	5.835	282085
Avg	2.6706	88098	5.849	281926.33

For 100 % Recovery

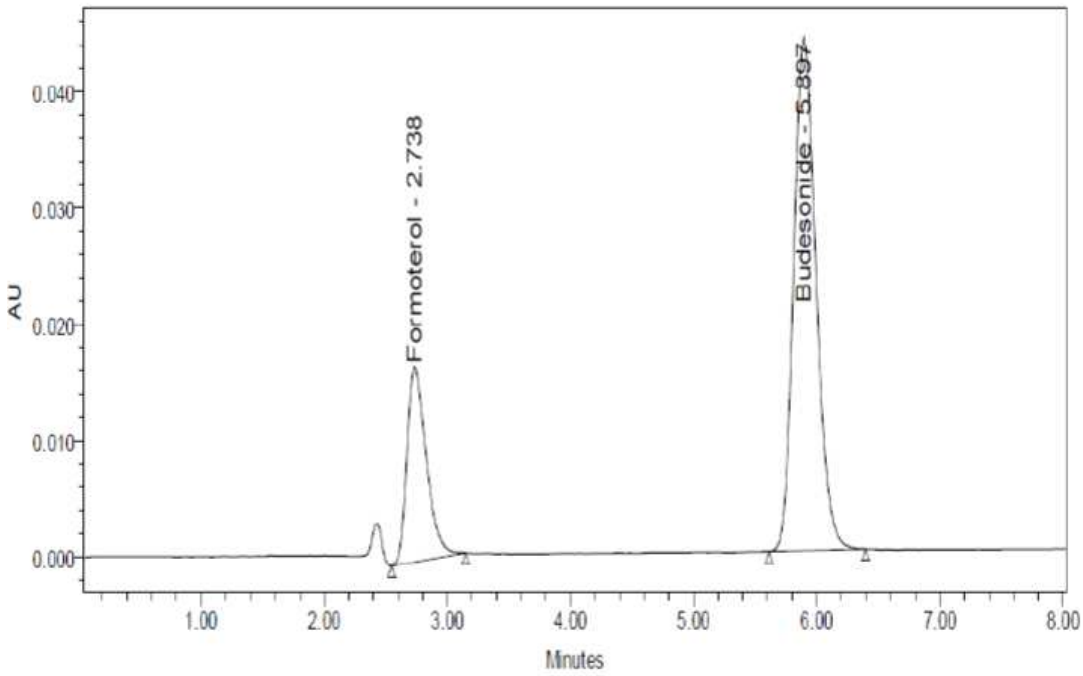

Fig. 6.25: Chromatogram of 100% recovery – injection 1

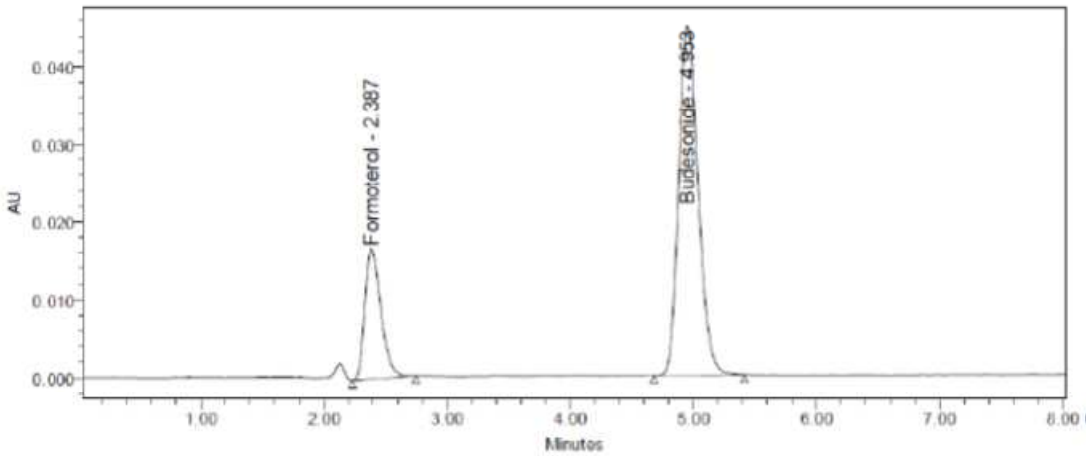

Fig. 6.26: Chromatogram of 100% recovery – injection 2

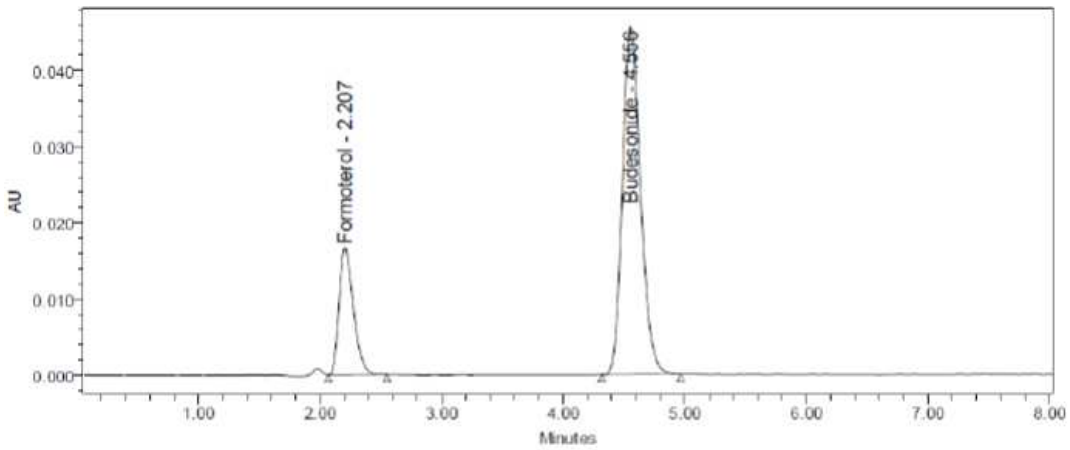

Fig. 6.27:Chromatogram of 100% recovery – injection 3

Table 6.26: Results for 100% Recovery

		FF	**BU**	
Injection				
	RT	**Area**	**RT**	**Area**
1	2.738	183067	5.897	552361
2	2.387	151053	4.953	490353
3	2.207	136633	4.556	469094
Avg	2.444	156917.66	5.135	503939.33

For 150 % Recovery

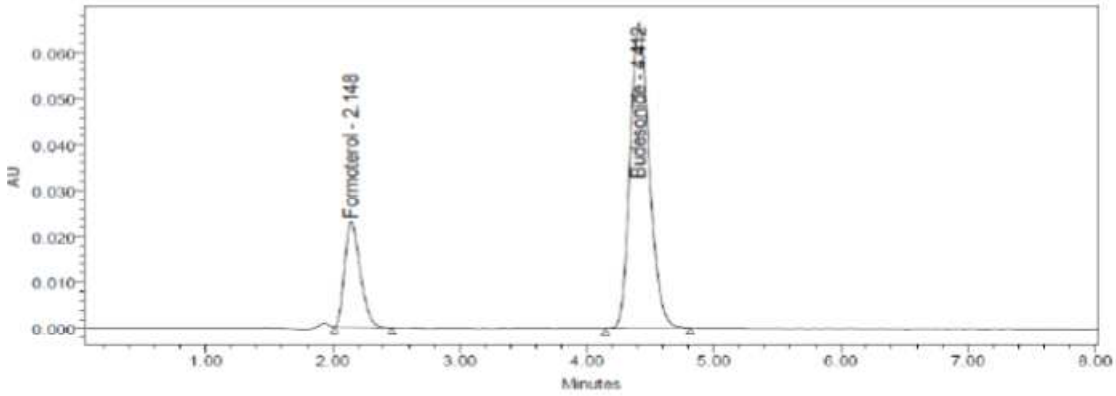

Fig. 6.28: Chromatogram of 150% recovery – injection 1

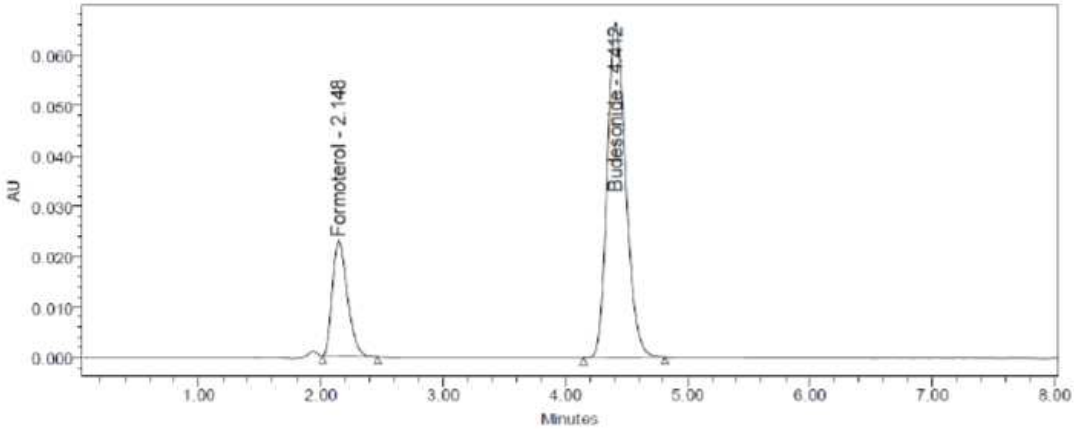

Fig. 6.29: Chromatogram of 150% recovery – injection 2

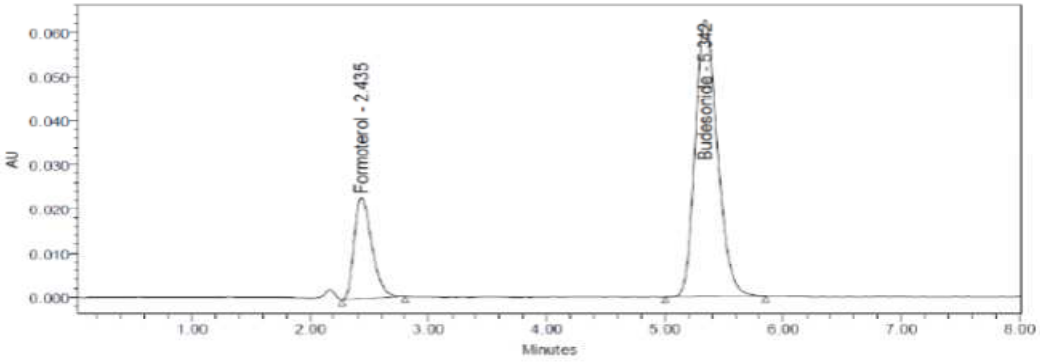

Fig.6.30: Chromatogram of 100% recovery – injection 3

Table 6.27: Results for 150% Recovery

Injection	FF		BU	
	RT	Area	RT	Area
1	2.148	196994	4.412	697285
2	2.148	196994	4.412	697285
3	2.435	235736	5.342	815472
Avg	2.243	209908	4.722	736680.666

Table 6.28:Results for Recovery of Formoterol Fumarate

Concentration	**Area**	**Amount added**(µg / mL)	**Amount found**(µg/ mL)	**% Recovery**	**% mean Recovery**
50	88098	50(µg/ mL)	49.26	98.52	
100	156917.66	100(µg/ mL)	99.75	99.75	98.82
150	209908	150(µg/ mL)	147.38	98.2	

Table 6.29: Results for 150% Recovery

Concentration	**Area**	**Amount added**(µg/ mL)	**Amount found**(µg/ mL)	**% Recovery**	**% mean Recovery**
50	281926	50(µg/ mL)	48.75	97.5	
100	503939	100(µg/ mL)	102.25	102.5	99.6
150	736680	150(µg/ mL)	148.36	98.8	

Acceptance criteria

The % recovery of Formoterol Fumarate and Budesonide should lie between 98% and 102%. The RSD of all the recovery values should not be more than 2.0%.

Observation

The percentage mean recovery of Formoterol Fumarate and Budesonide is and respectively and the results were found to be within the limits.

❖ Precision

Precision was determined by analyzing standard preparation of Formoterol Fumarate (50µg,ml) and Budesonide (2.5µgml) for six times. The chromatograms were recorded and the results were summarized in Table 6.30.

Table 6.30: Results for Precision

Injection	Formoterol Fumarate		Budesonide	
	RT	Area	RT	Area
1	2.092	132443	4.327	436949
2	2.093	130445	4.330	435877
3	2.094	128713	4.331	431699
4	2.094	128211	4.332	432385
5	2.095	132105	4.333	433739
6	2.096	126517	4.333	435272
Average		**1297389**		**434319.9**
SD		**2331.2**		**2058.8**
%RSD		**1.8**		**0.5**

Acceptance criteria

The % Relative standard deviation of Peak area of Formoterol Fumarate and Budesonide from the six replicate injections should be not more than 2.0%

Observation

Test results for Formoterol Fumarate and Budesonide are showing that the %RSD of Assay results are within limits. The results were shown in table 6.30.

❖ Limit of Detection (LOD)

The following formula was used calculate LOD

$$LOD = \frac{3.3\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The LOD for this method was found to be 0.655µg forFormoterolFumarate and 1.201µg for Budesonide

❖ Limit of Quatification

$$LOQ = \frac{10\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The LOQ for this method was found to be 1.9 µg for Formotero l Fumarate and 3.642 µg for Budesonide

❖ Robustness

Chromatographic conditions variation

To demonstrate the robustness of the method, prepared standard solution as per test method and injected in 5 replicate at different variable conditions like using different conditions like flow rate and temperature, wavelength, mobile phase organic composition. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. System suitability parameters were compared with that of method precision.

Acceptance criteria

The system suitability should pass as per the test method at variable conditions.

- **Variation in flow**

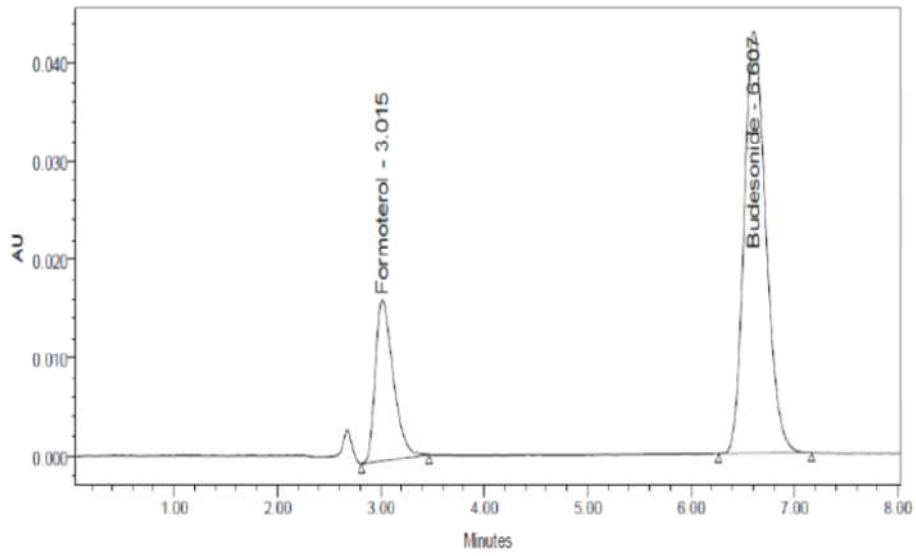

Fig. 6.31:Chromatogram of FF and BU for Robustness(Less flow 0.8 mL/min)

Table 6.31:Results of Formoterol Fumarate and Budesonide for Robustness (0.8ml/min)

Drug	RT(min)	Peak Area	TF	Efficiency	Resolution
FF	3.015	197661	1.40	2436.50	-
BU	6.607	625443	1.21	4740.46	10.08

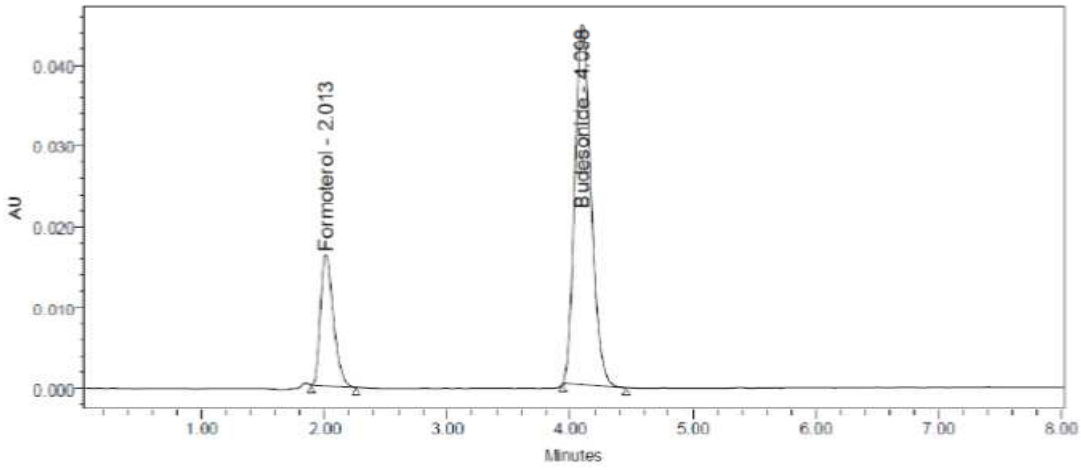

Fig. 6.32:Chromatogram of FormoterolFumarate and Budesonide for Robustness (More flow 1.2 mL/min)

Table 6.32: Results of FormoterolFumarate and Budesonide for Robustness (1.2 mL/min)

Drug	RT (min)	Peak area	TF	Efficiency	Resolution
FF	2.013	118857	1.35	2750.35	--
BU	4.098	413383	1.21	44983	9031

- **Variation in Wave Length**

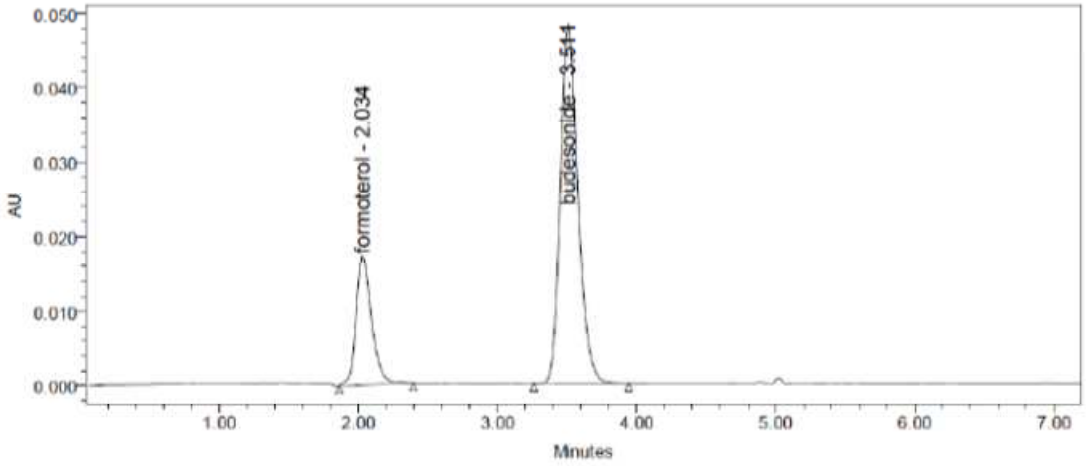

Fig. 6.33: Chromatogram ofFormoterolFumarate and Budesonide for Robustness (226nm)

Table 6.33:ResultsofFormoterolFumarate and Budesonide for Robustness (226 nm)

Drug	RT (min)	Peak Area	TF	Efficiency	Resolution
FF	2.034	133805	1.19	1708	-
BU	3.511	483920	1.21	3721	6.7

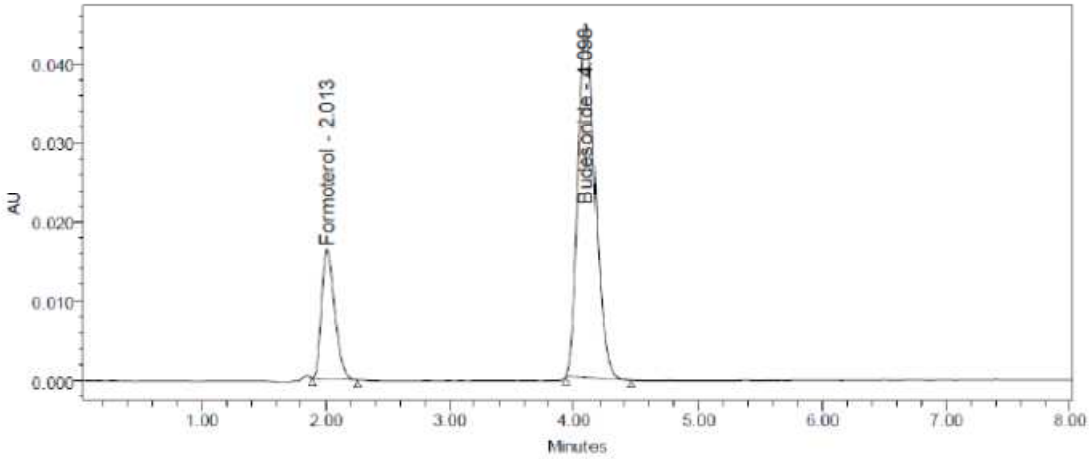

Fig.6.34:ChromatogramofFormoterolFumarate and Budesonide for Robustness (230nm)

Table.6.34:Results of Formoterol Fumarate and Budesonide for Robustness (230nm)

Drug	RT (min)	Peak Area	TF	Efficiency	Resolution
FF	2.013	118857	1.35	2750.35	-
BU	4.098	413383	1.21	4454.13	9.31

Variation of mobile phase organic composition

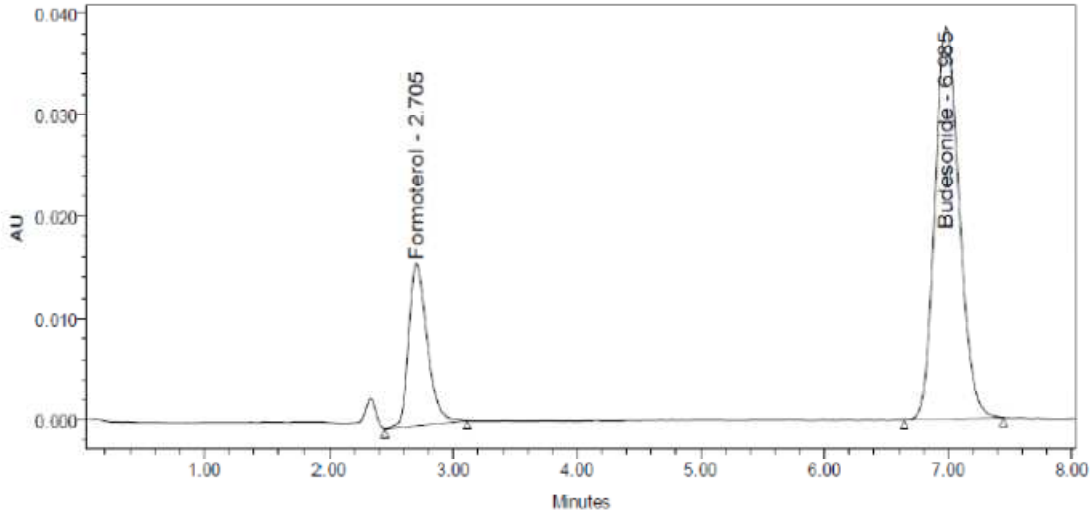

Fig. 6.35:Chromatogram of Formoterol Fumarate and Budesonide for robustness less organic

Table 6.35:Results ofFormoterolFumarate and Budesonide for Robustness (less organic)

Drug	RT (min)	Peak Area	TF	Efficiency	Resolution -
FF	2.705	166234	1.28	1624.81	-
BU	6.985	527144	1.15	6001.97	13.38

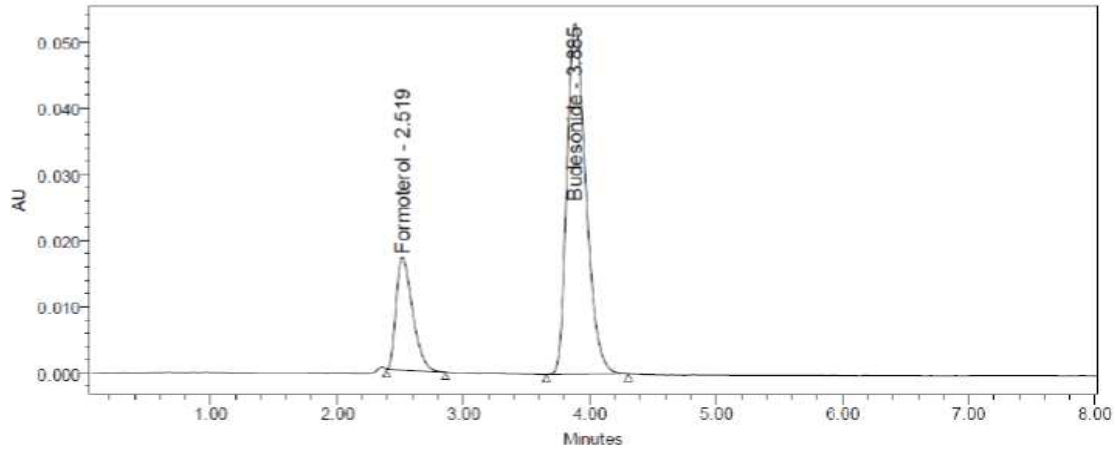

Fig. 6.36:Chromatogram of FormoterolFumarate and Budesonide forRobustness(More Organic)

Table 6.36: Summary Result of Robustness study

Parameter	Formoterol Fumarate (FF)		Budesonide (BU)	
	RT(min)	TF	RT(min)	TF
Flow Rate				
0.8 mL/min	3.015	1.40	6.607	1.21
1.0 mL/min	2.051	1.17	4.234	1.21
1.2 mL/min	2.013	1.35	4.098	1.21
Wavelength				
226 nm	2.034	1.19	3.511	1.32
228 nm	2.051	1.17	4.234	1.21
230 nm	2.013	1.35	4.098	1.21
Mobile Phase				
Less organic(55%) peak Area	2.705	1.28	6.985	1.85
organic (50%) peak Area	2.051	1.17	4.234	1.21
More organic (65%) peak area	2.519	1.49	3.885	1.31S

Observation

From the results of robustness performed variations in flow rate and wavelength and composition of Mobile phase . It was observed that not much variation in tailing factor was observed with deliberate changes in flow rate and wavelength and composition of mobile phase. The tailing factor was found to be within the limits for Formoterol Fumarate and Budesonide.

7. SUMMARY AND CONCLUSION

From the above observation data of the present research work related to new method development of Formoterol Fumarate and Budesonide was found satisfactory, simple, precise, accurate with good resolution, shorter retention time and among the other degradation products both Formoterol Fumarate and Budesonide were well separated with all accurate results. Low limit of quantization and limit of detection makes this method suitable for use in quality control. The less retention time obtained for the both drugs which reduces the run time enhances the usage of this method.

This is the first reported method for stability indicting simultaneous quantitative analysis of Formoterol Fumarate and Budesonide, and is a significant advance in chromatographic analysis of such pharmaceutical mixtures.. Forced degradation study results have shown good separation from degradation peaks. Hence we can clearly say that the proposed method is Economic, stable and truly novel validated method than the other reported methods

In the present developed method the acceptable validation parameters makes this method of analysis more acceptable for the routine analysis in quality control department in industries. Approved testing laboratories, bio-pharmaceutical and bio-equivalence studies and in clinical pharmacokinetic studies.

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